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(57) Abstract

Plants, particularly cereal plants which have modifications to their starch synthesising pathway contain a DNA specifying the enzyme soluble starch synthase which has the sequence SEQ ID NO 1 or SEQ ID NO 2 or SEQ ID NO 3. The inserted gene may be inserted in a sense or anti-sense construct. The alteration introduced by the inserted genes may be a greater or reduced ability to produce starch or starch which has a different fine structure such as a different pattern of branching.

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Exhibit 56

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MODIFICATION OF STARCH SYNTHESIS IN PLANTS

This invention relates to the alteration of the biosynthetic pathway which leads to production of starch in plants. By the term "alteration" we mean a change from normal of the amount or quality of the starch which the plant produces. More particularly, the invention relates to the isolation, purification and characterisation of the DNAs encoding several forms of the enzyme soluble starch synthase and the use of those DNAs through genetic modification of the plant genome to alter the starch production.

The invention also relates to novel plants having an improved ability to produce starch including an improved ability to produce structurally-altered starch.

Our previous studies have led to a new understanding of the metabolic pathway of starch synthesis in developing starch storing tissues (Keeling et al, 1988, Plant Physiology, 87:311-319; Keeling, 1989, ed. C.D. Boyer, J.C. Shannon and R.C. Harrison; pp.63-78, being a presentation at the 4th Annual Penn State Symposium in Plant Physiology).

Starch is an important end-product of carbon fixation during photosynthesis in leaves and is an important storage product in seeds and fruits. In economic terms, the starch produced by the edible portions of three grain crops, wheat, rice and maize, provide approximately two-thirds of the world's food calculated as calories.

Starch is synthesised in the plastid compartment, the chloroplast, in photosynthetic cells or the amyloplast in non- photosynthetic cells. The biochemical pathway of starch biosynthesis in leaves has been well-characterised (Figure 1). In contrast, little is known of the pathway of starch biosynthesis in storage organs.

Two principal methods for the control of gene expression are known. These are referred to in the art as "antisense downregulation" and "sense downregulation" or "cosuppression". Both of these methods lead to an inhibition of expression of the target gene. Overexpression is achieved by insertion of one or more than one extra copies of the selected gene. Other lesser used methods

involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene.

In antisense downregulation, a DNA which is complementary to all or part of the target gene is inserted into the genome in reverse orientation and without its translation initiation signal. The simplest theory is that such an antisense gene, which is transcribable but not translatable, produces mRNA which is complementary in sequence to mRNA product transcribed from the endogenous gene: that antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence: a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 40 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one plant species to be effective in another and obviates the need to construct antisense vectors for each individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It is the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the operability of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it has been used are on the market.

Both overexpression and downregulation are achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some overexpressing the target gene, some underexpressing. A population of plants produces by this method may then be screened and individual phenotypes isolated. As with antisense, the inserted sequence is lacking in a translation initiation signal. Another similarity with antisense is that the inserted sequence need not be a full length copy. Indeed, it has been found that the distribution of over- and under- expressing phenotypes is skewed in favour of underexpression and this is advantageous when gene inhibition is the desired effect. For overexpression, it is preferable that the inserted copy gene retain its translation initiation codon. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of this technology. It is well-established, used routinely in laboratories around the world and products in which it has been used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in Biotechnology and Genetic Engineering Reviews 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been described by Gray et.al., Plant Molecular Biology, 19: 69-87 (1992).

Gene control by any of the methods described requires insertion of the sense or antisense sequence, with appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

For dicotyledonous plants the most widely used method is Agrobacterium-mediated transformation. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium Agrobacterium tumefaciens, or the related Agrobacterium rhizogenes, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by Agrobacterium in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

The effectiveness of Agrobacterium is restricted to the host range of the microorganism and is thus restricted more or less to dicotyledonous plant species. In general monocotyledonous species, which include the important cereal crops, are not amenable to transformation by the Agrobacterium method. Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

In summary, then, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are

known. What remains, then is to identify genes whose regulation will be expected to have a desired effect, isolate them or isolate a fragment of sufficiently effective length, construct a chimeric gene in which the effective fragment is inserted between promoter and termination signals, and insert the construct into cells of the target plant species by transformation. Whole plants may then be regenerated from the transformed cells.

An object of the present invention is to provide DNAs encoding soluble starch synthases.

An further object of the invention is to provide novel plants having an increased capacity to produce starch and a capacity to produce starch with an altered fine structure.

According to the present invention there is provided cDNAs having the sequences of the inserts in plasmids pSSS6, pSSS10.1 and pSSS6.31 and sequences having sufficient similarity such that when inserted into the genome of an organism which produces starch, the synthesis of starch is altered.

The plasmid pSSS6 was deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 13th June 1994, under the Accession Number 40651.

The plasmids pSSS6.31 and pSSS10.1 were deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Numbers NCIMB 40679 and 40680 respectively.

The invention also provides the cDNAs, encoding soluble starch synthases which have the sequences SEQ-ID-NO-1, SEQ-ID-NO-2 AND SEQ-ID-NO-3.

The invention also provides transformed plants containing one or more copies of one or more of the said cDNAs in sense or antisense orientation.

The description which follows will describe a method for the isolation of the genes encoding soluble starch synthases from maize.

These DNAs can be used for the isolation of the corresponding genomic sequences. Either the cDNAs or the genes can then be used in studies leading to the

increase in starch yield. One possible application could be the use of these sequences to increase gene dosage of SSS in transformed crop plants to determine the contribution of SSS to the net regulation of starch biosynthesis, and to modify the levels of starch synthesised by the plant. The introduction of additional copies of SSS genes should produce greater levels of the enzyme in the amyloplasts. Increased gene expression may also be elicited by introducing multiple copies of enhancer sequences into the 5'-untranscribed region of SSS gene. If the enzyme is rate-limiting to starch biosynthesis, then the rate of starch biosynthesis would be expected to increase in the transformed plants. By virtue of this invention it will also be possible to alter the kinetic properties of the endopserm enzyme through protein engineering. Obviously a number of other parameters could also be improved. The present invention will now be described, by way of illustration, by the following Example and with reference to the accompanying drawings of which:

Figure 1 shows the reactions involved in the biosynthetic pathways of starch and glucose in leaves. The abbreviations used are: G-3-P,glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; Pi, orthophosphate; PPi, inorganic pyrophosphate. The reactions are catalysed by the following enzymes:

1) phosphoglycerate kinase/glyceraldehyde-3-phosphate dehydrogenase
2) triose-phosphate isomerase
3) aldolase
4) fructose-1,6-bisphosphatase
5) hexose phosphate isomerase
6) phosphoglucomutase
7) ADP-glucose pyrophosphorylase
8) starch synthase
9) UDP-glucose pyrophosphorylase
10) sucrose phosphate synthase
11) sucrose phosphatase
12) orthophosphate/triose phosphate translocator
13) inorganic pyrophosphatase

Figure 2 shows the proposed metabolic pathway of starch biosynthesis in wheat endosperm (Keeling et. al. 1988). The abbreviations used are the same as in Figure 1. The reactions are catalysed by the following enzymes:

1) sucrose synthase
2) UDP-glucose pyrophosphorylase
3) hexokinase
4) phosphoglucomutase
5) hexose-phosphate isomerase
6) ATP-dependent phosphofructokinase
7) PPi-dependent phosphofructokinase
8) aldolase
9) triose-phosphate isomerase
10) hexose-phosphate translocator
11) ADP-glucose pyrophosphorylase
12) starch synthase
13) sucrose phosphate synthase
14) sucrose phophatase

USE OF SOLUBLE STARCH SYNTHASE OR BRANCHING ENZYME

Using standard cloning techniques, the SSS genes may be isolated. The source of the genes was a US yellow-dent corn line of Zea mays, from which the enzyme protein was purified. Endosperms from the maize line were homogenised in a buffer which maintains the SSS in active form.

Purification of the SSS from maize has been achieved by a combination of ammonium sulphate precipitation, DEAE-cellulose chromatography, gel-filtration, phenyl Superose and FPLC using a Mono-Q column. This results in several hundred-fold purification with yields up to 5%. The SSS polypeptide was a single subunit of molecular weight 76kDa. Other SSS polypeptides were present in a US dent inbred line at around 60kDa, 70kDa and 105kDa molecular weight.

Ammonium sulphate precipitation of SSS I is best achieved using 10-35% ammonium sulphate which produces a translucent SSS-enriched pellet which is next dialysed and further fractionated using DEAE-cellulose ion-exchange chromatography (2.5 x 5cm column). SSS was eluted with a 150 ml gradient of KCl (0-0.6M) and fractions collected. These steps increase specific activities by up to 12-fold. The DEAE peak fractions were concentrated by precipitation with ammonium sulphate (40%) and the resulting pellet dissolved in buffer and fractionated on a Sephacryl S-200 column (2.5 x 100 cm) equilibrated with buffer and fractions collected. These steps increase specific activities by up to 8-fold. A Phenyl-Superose column was equilibrated with buffer containing ammonium sulphate. SSSI did not bind and was present in the pass-through fraction. These steps increase specific activities by up to 2-fold. Finally, a Mono-Q column was equilibrated with buffer and charged with the Phenyl-Superose pass-through fraction. The enzymes were eluted from the column using a 12 ml linear gradient of 0-0.5 M KCl and fractions collected. These steps increase specific activities by up to 5-fold.

In the final purification step the SSS preparations were loaded on to SDS PAGE gels. The bands corresponding to the SSS polypeptides were cut out and eluted. The polypeptide was sequenced using standard amino acid sequencing techniques.

In order to produce a pure antigen for antibody production, we decided to use starch granules as our starting-point for isolation of SSS proteins. Kernels were homogenised in buffer by grinding in a Waring blender. The homogenate filtered through miracloth and centrifuged. After discarding the supernatant and the discoloured material that overlays the white starch pellet, the pellet was washed twice with buffer and centrifuged. Starch was washed a final time with chilled acetone and following centrifugation, dried under a stream of air before storing at -20C. Granule protein was extracted by boiling 1.4 g starch for 10 minutes in 50ml SDS-PAGE sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 62.5 mM Tris/HCl, pH 6.8) which lacked bromophenol blue. After cooling and centrifugation at 25,000 g at 4C for 15 minutes, the supernatant was mixed with an equal volume of 30% TCA and allowed to stand at 4C for 1 hour. The solution was centrifuged again and pellet washed twice with 10 ml acetone before resuspension in 1.4 ml SDS-PAGE sample buffer. Following separation of granule-derived proteins by SDS-PAGE, the SSS proteins (eg 60kDa, 76kDa etc) bands were electroeluted and used as antigen (three 50ug doses at 4-week intervals, in New Zealand white rabbits) to generate polyclonal antibodies in a rabbit. The antibodies were then tested for specificity to the SSS

polypeptides. Antibodies were monospecific and have enabled a thorough analysis of enzyme activities and expression studies.

N-terminal amino acid sequences were also obtained from the polypeptides. These proteins were shown to be identical with soluble proteins on the basis of (i) N-terminal sequences to the SSSs as purified by conventional means and sequenced were identical to the granule derived proteins, and (ii) protease digests gave peptide maps which were also identical.

Amino acid sequencing of the maize SSS polypeptide has yielded the following partial sequences:

N-terminal... C V A E L S R E G P A P R

Internal sequences:

K N Y A N A F Y T E T H I
E L G G Y I Y G Q N D M F V V N N D H A S L V P V L L A A K Y I R
E V T T A E G G S G L N E L L
G K I D N T V V V A S E Q D S Y

The antibodies may be used to screen a maize endosperm cDNA library for clones derived from the mRNAs for SSS in an in vitro transcription/ translation system. Synthetic oligos may be constructed and used to screen maize endosperm cDNA library. The SSS sequence may be compared to the amino acid sequence of pea SSS I and SSS II published by Dry et al (1991, Plant Journal 2:193-202) or rice SSS published by Baba et al (1993, Plant Physiology 103, 565-573). Interestingly, the clone obtained from rice SSS is not correctly identified. The N-terminal sequence AELSREG is stated to be part of the transit peptide sequence of the rice clone. This error must have occurred because of protein isolation problems from rice kernels: presumably a portion of the protein was cleaved prior to isolation. Using our N-terminal sequence, the corrected molecular weight of the rice clone is around 69kDa and not 55 or 57kDa as suggested by Baba et al.

cDNA LIBRARY SCREENING AND ISOLATION OF SSS cDNA CLONES

RNA was extracted from from 21 DAP endosperm (obtained from the inbred line B73) after removal of pericarp and embryo. The library consisted of ~900,000 recombinant clones. A probe for granule bound starch synthase was generated using PCR and used to screen an aliquot of the library, ~500,000 recombinants. This screening yielded approximately 200 positive signals. Isolation and sequencing of a number showed them to be full length GBSS cDNA clones.

An oligonucleotide was synthesised to N-terminal sequence obtained from the purified SSS protein and used to screen the same aliquot of library as that used for the GBSS screening. No positive signals were obtained. A long oligonucleotide probe was then synthesised to the ADP-ADPG binding region and following sequence, based on a comparison of the sequences published for pea SSS, rice SSS and maize GBSS.

The sequence of the oligonucleotide was GGT/C GGA/G CTA/T GGAGATGTTTGTGGA/T TCACTCCCAATTGCTCTT/G GCTCTTCGTGGA/T CATCGTGTG/T ATGGTTGT. Fifteen strong signals were obtained,all were picked, of these ten plaque purified after two rounds of purification. Restriction analysis of all ten showed them to fall into two classes. Sequence analysis showed both classes to be starch synthases.

Screening of a maize seedling library (Clontech) gave positive signals using 5' probes from one class of clones only.

A cDNA library from the inbred line W64A was screened and full length clones were isolated as judged by comparison with N-terminal sequence.

CHARACTERISATION OF cDNA CLONES

The isolated cDNAs were sequenced and are given herewith as SEQ-ID-NO-1, NO-2 and NO-3.

For comparison, the deduced amino acid sequences are shown here with the sequences obtained directly from the protein:-

CVAELSREGPAPR	peptide derived
CVAELSREGPAPR 6.4	deduced cDNA
KXYANAFYTETHI	peptide derived
KNYANAFYSEKHI 10.52	deduced cDNA

EVTTAEGGSGLNELL peptide derived

EVTTAEGGQGLNELL deduced cDNA 10.52

ELGGYIYGANXMFVVNXXHASLVPVLLAAKY peptide derived

ELGGYIYGQNCMLVVNDWHASLEPVLLAAKY deduced cDNA 10.52

GKIDNTVVVASEQDSY peptide derived

GSIDNTVVVASEQDSE deduced cDNA10.52

Isolated from soluble 76kDa protein.....
GLVVTRDRDRIQ-VASNR peptide derived

GAVVTADRIVTVSKGYS deduced cDNA 10.52

Clone SSS6.31 contained none of these internal sequences. The motif for the binding-site of ADPG and ADP, thought to be part of the active site of starch synthases is found in all clones near to the 5' end and is followed by the highly conserved sequence on which the oligonucleotide probe was based. The highly conserved domain SRFEPCGLNQLYAMXYGTXXXXXXXXGGLRDTV is present in SSS10.52 but is slightly modified in SSS6.31 in that the EPC motif is replaced with an AG motif.

Expression of maize starch synthases in Escherichia coli BL21(DE3).

These SSS clones have been transfected into E.coli. The SSS activity was measured and are reported in the Table below.

Plasmids	Maize starch synthase genes	N-terminus	Protein	Specific

			(mg/mL)	Activities* (units/mg Protein)
pET21a	Native plasmid	<no insert>	1.8	.009
pEXS-3a	MSSSII (MSSS631)	GENVMNVIV V	2.8	0.069
pEXS-8	MSSSI (MSSS6-4)	CVAELSREGP	1.9	0.097
pEXS-9	MSSSIII (MSSS5.6)	GSVGAALRSY	1.8	0.515
pEXS-wx	MGBSS (waxy)	ASAGMNVVF V	2.0	0.033

- One unit activity is defined as one mmol glucose incorporated into a-1,4 glucan per minute at 25^{0}C using 5 mg/mL glycogen as primer.

GENE CONSTRUCTS FOR TRANSFORMATION

The gene constructs require the presence of an amyloplast transit peptide to ensure its correct localisation in the amyloplast. It is believed that chloroplast transit peptides have similar sequences but other potential sources are available such as that attached to ADPG pyrophosphorylase (Plant Mol. Biol. Reporter (1991) 9, 104-126). Other potential transit peptides are those of small subunit RUBISCO, acetolactate synthase, glyceraldehyde-3P-dehydrogenase and nitrite reductase. For example,

Consensus sequence of the transit peptide of small subunit RUBISCO from many genotypes has the sequence:

MASSMLSSAAV¾ATRTNPAQAS MVAPFTGLKSAAFPVSRK QNLDITSIA SNGGRVQC

and the corn small subunit RUBISCO has the sequence:

MAPTVMMASSAT-ATRTNPAQAS AVAPFQGLKSTASLPVARR SSRSLGNVA SNGGRIRC

The transit peptide of leaf starch synthase from corn has the sequence:

MA ALATSQLVAT RAGLGVPDAS TFRRGAAQGL RGARASAAAD TLSMRTASARA APRHQQQARR GGRFPSLVVC

The transit peptide of leaf glyceraldehyde-3P- dehydrogenase from corn has the sequence:

MAQILAPS TQWQMRITKT SPCATPITSK MWSSLVMKQT KKVAHSAKFR VMAVNSENGT

The putative transit peptide from ADPG pyrophosphorylase from wheat has the sequence: RASPPSESRA PLRAPQRSAT RQHQARQGPR RMC

It is possible however to express the genes constitutively using one of the well-known constitutive promoters such as CaMV35S but there may be biochemical penalties in the plant resulting from increased starch deposition throughout the entire plant. Deposition in the endosperm is much preferred.

Possible promoters for use in the invention include the promoters of the starch synthase gene, bound starch synthase gene, endopserm hsp70 gene, ADPG pyrophosphorylase gene, and the sucrose synthase gene.

FOR TESTING GENE EXPRESSION IN ENDOSPERM TISSUE:				
Plasmid name	**Promoter**	**Intron**	**Targetting**	**Gene**
pHKH1	CaMV35S	adh1	WxTrPep	GUS
pSh1PIGN	CaMV35S	adh1	WxTrPep	GUS
pSh2PIGN	CaMV35S	adh1	WxTrPep	GUS

FOR TESTING IN SUSPENSION CELL CULTURES:				
Plasmid name	**Promoter**	**Intron**	**Targetting**	**Gene**
p***1	CaMV35S	Sh1	WxTrPep	GUS
p***2	CaMV35S	adh1	WxTrPep	GUS

FULL VECTORS FOR PLANT TRANSFORMATION				
Plasmid name	**Promoter**	**Intron**	**Targetting**	**Gene**
p***21	Waxy	Sh1	WxTrPep	SSS and/or BE
p***22	Waxy	Adh1	WxTrPep	SSS and/or BE
p***23	Sh1	Sh1	WxTrPep	SSS and/or BE
p***24	Sh1	Adh1	WxTrPep	SSS and/or BE
p***25	Sh2	Sh1	WxTrPep	SSS and/or BE
p***26	Sh2	Adh1	WxTrPep	SSS and/or BE
p***27	hsp70	Sh1	WxTrPep	SSS and/or BE
p***28	hsp70	Adh1	WxTrPep	SSS and/or BE

TRANSFORMATION

(i) Insertion of extra copies of the gene

Maize genomic DNAs isolated as above may subsequently be transformed into either protoplasts or other tissues of a maize inbred line or population. The existing gene promoters ensure that the extra genes are expressed only in the developing endosperm at the correct developmental time. The protein sequences likewise ensure that the enzymes are inserted into the amyloplast.

Transgenic maize plants are regenerated and the endosperms of these plants are tested for increased SSS enzyme activity. The kernels are also tested for enhanced rate of starch synthesis at different temperatures. The plants are then included in a breeding programme to produce new maize hybrids with higher rates of starch synthesis at temperatures above the normal optimum.

(ii) Insertion of genes specifying SSS

This is also achieved by standard cloning techniques. The source of the temperature-stable forms of the SSS genes is any organism that can make starch or glycogen. Potential donor organisms are screened and identified as described above. Thereafter there are two approaches:

(a) via enzyme purification and antibody/sequence generation using the protocol described above.

(b) using SSS cDNAs as heterologous probes to identify the genomic DNAs for SSS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this instance it is necessary to make gene constructs for transformation which contain the regulatory sequences from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct developmental time (eg. ADPG pyrophosphorylase).

Gene constructs used to transform plants requires the regulatory sequences from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct development time (eg. ADPG pyrophosphorylase).

Furthermore the gene constructs also requires a suitable amyloplast transit-peptide sequence such as from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to censure expression of the amyloplast at the correct developmental time (eg, ADPG pyrophosphorylase).

Genetic protein engineering techniques may also be used to alter the amino acid sequence of the SSS enzymes to impart higher temperature optima for activity. The genes for SSS may be cloned into a bacteria which relies on these enzymes for survival. Selection for bacteria surviving at evaluated temperatures enables the isolation of mutated thermostable enzyme forms. Transformation of maize with the altered genes is carried out as described above.

(iii) Changing the ratios of activities of the isoforms of enzymes SSS

This is also achieved by standard cloning techniques. The source of the SSS genes is maize using the protocol described above. Plants are then transformed by insertion of extra gene copies of the isoforms of SSS enzymes and/or by insertion of anti- sense gene constructs. The gene promoters and other regulatory sequences may also be altered to achieve increased amounts of the enzyme in the recipient plant.

(iv) Insertion of a gene or genes specifying SSS with activities which effect a change in the fine structure of the starch.

This is also achieved by standard cloning techniques. The source of the special forms of the SSS is any organism that can make starch. Potential donor organisms are screened and identified as described above. Thereafter there are two approaches:

(a) via enzyme purification and antibody/sequence generation using the protocol described above.

(b) using SSS cDNAs as heterologous probes to identify the genomic DNAs for SSS in libraries from the organism concerned. The gene transformation, plant regeneration and testing protocols are as described above. In this instance it is necessary to make gene

constructs for transformation which contain the regulatory sequences from maize endosperm SSS or another maize endosperm starch synthesis pathway enzyme to ensure expression in endosperm at the correct developmental time (eg, ADPG pyrophosphorylase).

Full length clone sequences

SEQ-ID-NO1; DNA; 2992 BP.

CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS1052 and SSS64

SQ SEQUENCE 2992 BP; 758 A; 655 C; 801 G; 776 T; 2 OTHER;

GAATTCGCGG CCGCCTTATT TCTGGTTGGC CACATACATC ATCCAAAAAA CTTTATTATT

GAATTACAAC TAATAAGCAA TCTAAAAGAG GGCACCACCA ATGATGTGTT GTTGGTAGGA

GGCCGCTGGG TCTGTCAAAG CAAGTTGGAC AAAGGGCAAC AATTGTTGTA GTTGTAAGAG

GGTTGCGGGG TTAGCCGCAA ACTGCTGGTA GAAAGGCAGC AACTGTTGCT GTGTCAAGAA

GGAAGCACGG TTTGCTGCAG CTGTTGTGCC CTGATGGTTT GTACCAATGA CTGCACCAAA

GATAGGGCTG GCGATTGTTG AAACAACAAG GGCGATAAAG GTATGTTGCT TGCTGCGATT

GCTTGTTGAA GCCTATATGG TTGAAGAGCT GGGTTTTCAC ATATTGAAGC TATAATTGAT

GGAAGGTATG GGGGAAGAAG GGAAGCTATA GGAGCTTGTG AGCATTGAGG GAAAATTGTC

GCGTTAGCAA CACATGTAGA AAGAGCAAGG AGCATAAGGA GGGAAAATAT CTTGGTCGCC

ATTGTTGCGC GCGATCCACG GCCCCCCCCC CCCGCGCTCC TGTCTGCTCT CCCTCTCCGC

AATGGCGACG CCCTCGGCCG TGGGCGCCGC GTGCCTCCTC CTCGCGCGGG NCG CCTGGCC

GGCCGCCGTC GGCGACCGGG CGCGCCCGCG GAGGCTCCAG CGCGTGCTGC GCCGCCGGTG

CGTCGCGGAG CTGAGCAGGG AGGGGCCCGC GCCGCGCCCG CTGCCACCCG CGCTGCTGGC

GCCCCCGCTC GTGCCCGGCT TCCTCGCGCC GCCGGCCGAG CCCACGGGTG AGCCGGCATC

GACGCCGCCG CCCGTGCCCG ACGCCGGCCT GGGGGACCTC GGTCTCGAAC CTGAAGGGAT

TGCTGAAGGT TCCATCGATA ACACAGTAGT TGTGGCAAGT GAGCAAGATT CTGAGATTGT

GGTTGGAAAG GAGCAAGCTC GAGCTAAAGT AACACAAAGC ATTGTCTTTG TAACCGGCGA

AGCTTCTCCT TAATCGAAAG TCTGGGGGTC TAGGAGATGT TTGTGGTTCA TTGCCAGTTG

CTCTTGCTGC TCGCGGTCAC CGTGTGATGG TTGTAATGCC CAGACATTTA AATGGTACCT

CCGATAAGAA TTATGCAAAT GCATTTTACT CAGAAAAACA CATTCGGATT CCATTCTTTG

GCGGTGAACA TGAAGTTACC TTCTTCCATG AGTATAGAGA TTCAGTTGAC TGGGTGTTTG

TTGATCATCC CTCATATCAC AGACCTGGAA ATTTATATGG AGATAAGTTT GGTGCTTTTG

GTGATAATCA GTTCAGATAC ACACTCCTTT GCTATGCTGC ATGTGAGGCT CCTTTGGTCC

TTGAATTGGG AGGATATATT TATGGACAGA ATTGCATGTT GGTTGTCAAT GATTGGCATG

CCAGTCTAGA GCCAGTCCTT CTTGCTGCAA AATATAGACC ATATGGTGTT TATAAAGACT

CCCGCAGCAT TCTTGTAATA CATAATTTAG CACATCAGGG TGTAGAGCCT GCAAGCACAT

ATCCTGACCT TGGGTTGCCA CCTGAATGGT ATGGAGCTCT GGAGTGGGTA TTCCCTGAAT

GGGCGAGGAG GCATGCCCTT GACAAGGGTG AGGCAGTTAA TTTTTTGAAA GGTGCAGTTG

TGACAGCAGA TCGAATCGTG ACTGTCAGTA AGGGTTATTC ATGGGAGGTC ACAACTGCTG

AAGGTGGACA GGGCCTCAAT GAGCTCTTAA GCTCCAGAAA GAGTGTATTA AACGGAATTG

TAAATGGAAT TGACATTAAT GATTGGAACC CTGCCACAGA CAAATGTATC CCCTGTCATT

ATTCTGTTGA TGACCTCTCT TGAAAGGCTA AATGTAAAGG TGCATTGCAG AAGGAGCTGG

GTTTACCTAT AAGGCCTGAT GTTCCTCTGA TTGGCTTTAT TGGAAGATTG GATTATCAGA

AAGGCATTGA TCTCATTCAA CTTATCATAC CAGATCTCAT GCGGAAGAAT GTTCAA TTTG

TCATGCTTGG ATCTGGTGAC CCAGAGCTTG AAGATTGGAT GAGATCTACA GAGTCGATCT

TCAAGGATAA ATTTCGTGGA TGGGTTGGAT TTAGTGTTCC AGTTTCCCAC CGAATAACTG

CGGCTGGCGA TATATTGTTA ATGCCATCCA GATTCGAACC TTGTGGTCTC AATCAGCTAT

ATGCTATGCA GTATGGCACA GTTCCTGTTG TCCATGCAAC TGGGGGCCTT AGAGATACCG

TGGAGAACTT CAACCCTTTC GGTGAGAATG GAGAGCAGGG TACAGGGTGG GCATTCGCAC

CCCTAACCAC AGAAAACATG TTTGTGGACA TTGCGAACTG CAATATCTAC ATACAGGGAA

CACAAGTAAT AATGGGAAGG GCTAATGAAG CCAGGCATGT CAAAAGAGTT CACGTGGGAC

CATGCCGCTG AACAATACGA ACAAATCTTC CAGTGGGCCT TCATCGGATC GACCCGATGT

TCAATGGAAA AAAGGGACCA AAGTTGGTTG GTTCCTTGAA GATTATCAGT TCATCATCCT

ATAGTAAGCT GAATGATGAA AGAAAACCCC TGTACATTAC ATGGAAGGCA GACCGGCTAT

TGGCTCCATT GCTCCAATGT CTGCTTTGGC TGCCTTGCCT CGATGGACCG GATGCAGTGA

GGAATCCAGN CGAACGACAG TTTTGAAGGA TAGGAAGGGG AGCTGGAAGC AGTCACGCAG

GCAGGCAAGC CTTCGCCGTT AATTCATATG GAACAAGCTG GAGTCAGTTT CTGCTGTGCC

ACTCACTGTT TACCTTAAGA TTATTACCTG TGTTGTTCTC CTTTGCTCGT TAGGGCTGAT

AACATAATGA CTCATTAAGA ATATAATTCA CTCTGCCTCG TTTTTAATCT TAAGTGAAGT

CGAGATCTAC TTCGTCATTC CTTCCCCGTT TAAAAAAAAA AAAAAAAAAA AA

SEQ-ID-NO2; DNA; 2085 BP.

CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS CLONE 6.31

SQ SEQUENCE 2085 BP; 456 A; 521 C; 629 G; 479 T; 0 OTHER;

AACGCCGCAT TGGCACGTTG AGATCAAGTC CATCGTCGCC GCGCCGCCGA CGAGCATAGT

GAAGTTCCCA GGGCGCGGGC TACAGGATGA TCCTTCCCTC TGGGACATAG CGCCGGAGAC

TGTCCTCCCA GCCCCGAAGC CACTGCATGA ATCGCCTGCG GTTGACGGAG ATTCAAATGG

AATTGCACCT CCTACAGTTG AGCCATTAGT ACAGGAGGCC ACTTGGGATT TCAAGAAATA

CATCGGTTTT GACGAGCCTG ACGAAGCGAA GGATGATTCC AGGGTTGGTG CAGATGATGC

TGGTTCTTTT GAACATTATG GGACAATGAT TCTGGGCCTT TGTGGGGAGA ATGTTATGAA

CGTGATCGTG GTGGCTGCTG AATGTTCTCC ATGGTGCAAA ACAGGTGGTC TTGGAGATGT

TGTGGGAGCT TTACCCAAGG CTTTAGCGAG AAGAGGACAT CGTGTTATGG TTGTGGTACC

AAGGTATGGG GACTATGTGG AAGCCTTTGA TATGGGAATC CGGAAATACT ACAAAGCTGC

AGGACAGGAC CTAGAAGTGA ACTATTTCCA TGCATTTATT GATGGAGTCG ACTTTGTGTT

CATTGATGCC TCTTTCCGGC ACCGTCAAGA TGACATATAT GGGGGAAGTA GGCAGGAAAT

CATGAAGCGC ATGATTTTGT TTTGCAAGGT TGCTGTTGAG GTTCCTTGGC ACGTTCCATG

CGGTGGTGTG TGCTACGGAG ATGGAAATTT GGTGTTCATT GCCATGAATT GGCACACTGC

ACTCCTGCCT GTTTATCTGA AGGCATATTA CAGAGACCAT GGGTTAATGC AGTACACTCG

CTCCGTCCTC GTCATACATA ACATCGGCCA CCAGGGCCGT GGTCCTGTAC ATGAATTCCC

GTACATGGAC TTGCTGAACA CTAACCTTCA ACATTTCGAG CTGTACGATC CCGTCGGTGG

CGAGCACGCC AACATCTTTG CCGCGTGTGT TCTGAAGATG GCAGACCGGG TGGTGACTGT

CAGCCGCGGC TACCTGTGGG AGCTGAAGAC AGTGGAAGGC GGCTGGGGCC TCCACGACAT

CATCCGTTCT AACGACTGGA AGATCAATGG CATTCGTGAA CGCATCGACC ACCAGGAGTG

GAACCCCAAG GTGGACGTGC ACCTGCGGTC GGACGGCTAC ACCAACTACT CCCTCGAGAC

ACTCGACGCT GGAAAGCGGC AGTGCAAGGC GGCCCTGCAG CGGGACGTGG GCCTGGAAGT

GCGCGACGAC GTGCCGCTGC TCGGCTTCAT CGGGCGTCTG GATGGACAGA AGGGCGTGGA

CATCATCGGG GACGCGATGC CGTGGATCGC GGGGCAGGAC GTGCAGCTGG TGATGCTGGG

CACCGGCCCA CCTGACCTGG AACGAATGCT GCAGCACTTG GAGCGGGAGC ATCCCAACAA

GGTGCGCGGG TGGGTCGGGT TCTCGGTCCT AATGGTGCAT CGCATCACGC CGGGCGCCAG

CGTGCTGGTG ATGCCCTCCC GCTTCGCCGG CGGGCTGAAC CAGCTCTACG CGATGGCATA

CGGCACCGTC CCTGTGGTGC ACGCCGTGGG CGGGCTCAGG GACACCGTGG CGCCGTTCGA

CCCGTTCGGC GACGCCGGGC TCGGGTGGAC TTTTGACCGC GCCGAGGCCA ACAAGCTGAT

CGAGGTGCTC AGCCACTGCC TCGACACGTA CCGAAACTAC GAGGAGAGCT GGAAGAGTCT

CCAGGCGCGC GGCATGTCGC AGAACCTCAG CTGGGACCAC GCGGCTGAGC TCTACGAGGA

CGTCCTTGTC AAGTACCAGT GGTGAACCCT CCGCCCTCCG CATCAATATC TTCGGTTTGA

TCCCATTGTA CATCGCCCTT TGACGGTCTC GGTGAAGAAC TTCATATGCA GTGCCGTGCT

GGGGCGGTAG CAGTACTATG GGATTGCATT GAGCTGTGTC ACTATGTGCT TTCGACAGGA

CAGTAGTGAA GGTTCTATGC AAGTTTATTT TTTTTTTCAT TACTGATATT TGGAATGTCA

ACACAATAAA TAACTACTAT GTGTTTCGTA AGTAAAAAAA AAAAA

SEQ-ID-NO3: 2478 bp DNA 04-DEC-1995

CC NOTE: ORIGINAL SEQUENCE NAME WAS SSS56

SUMMARY #Molecular-weight 89141 #Length 826 #Checksum 2983

BASE COUNT 347 A 276 C 533 G 290 T

ORIGIN

1 GCNGCNGCNT GGTRRGCNYT NGTNCARGCN GARGCNGCNG TNGCNTRRGG NATHCCNATG

61 CCNGGNGCNA THWSNWSNWS NWSNWSNGCN TTYYTNYTNC CNGTNGCNWS NWSNWSNCCN

121 MGNMGNMGNM GNGGNWSNGT NGGNGCNGCN YTNMGNWSNT AYGGNTAYWS NGGNGCNGAR

181 YTNMGNYTNC AYTGGGCNMG NMGNGGNCCN CCNCARGAYG GNGCNGCNWS NGTNMGNGCN

241 GCNGCNGCNC CNGCNGGNGG NGARWSNGAR GARGCNGCNA ARWSNWSNWS NWSNWSNCAR

301 GCNGGNGCNG TNCARGGNWS NACNGCNAAR GCNGTNGAYW SNGCNWSNCC NCCNAAYCCN

361 YTNACNWSNG CNCCNAARCA RWSNCARWSN GCNGCNATGC ARAAYGGNAC NWSNGGNGGN

421 WSNWSNGCNW SNACNGCNGC NCCNGTNWSN GGNCCNAARG CNGAYCAYCC NWSNGCNCCN

481 GTNACNAARM GNGARATHGA YGCNWSNGCN GTNAARCCNG ARCCNGCNGG NGAYGAYGCN

541 MGNCCNGTNG ARWSNATHGG NATHGCNGAR CCNGTNGAYG CNAARGCNGA YGCNGCNCCN

601 GCNACNGAYG CNGCNGCNWS NGCNCCNTAY GAYMGNGARG AYAAYGARCC NGGNCCNYTN

661 GCNGGNCCNA AYGTNATGAA YGTNGTNGTN GTNGCNWSNG ARTGYGCNCC NTTYTGYAAR

721 ACNGGNGGNY TNGGNGAYGT NGTNGGNGCN YTNCCNAARG CNYTNGCNMG NMGNGGNCAY

781 MGNGTNATGG TNGTNATHCC NMGNTAYGGN GARTAYGCNG ARGCNMGNGA YYTNGGNGTN

841 MGNMGNMGNT AYAARGTNGC NGGNCARGAY WSNGARGTNA CNTAYTTYCA YWSNTAYATH

901 GAYGGNGTNG AYTTYGTNTT YGTNGARGCN CCNCCNTTYM GNCAYMGNCA YAAYAAYATH

961 TAYGGNGGNG ARMGNYTNGA YATHYTNAAR MGNATGATHY TNTTYTGYAA RGCNGCNGTN

1021 GARGTNCCNT GGTAYGCNCC NTGYGGNGGN ACNGTNTAYG GNGAYGGNAA YYTNGTNTTY

1081 ATHGCNAAYG AYTGGCAYAC NGCNYTNYTN CCNGTNTAYY TNAARGCNTA YTAYMGNGAY

1141 AAYGGNYTNA TGCARTAYGC NMGNWSNGTN YTNGTNATHC AYAAYATHGC NCAYCARGGN

1201 MGNGGNCCNG TNGAYGAYTT YGTNAAYTTY GAYYTNCCNG ARCAYTAYAT HGAYCAYTTY

1261 AARYTNTAYG AYAAYATHGG NGGNGAYCAY WSNAAYGTNT TYGCNGCNGG NYTNAARACN

1321 GCNGAYMGNG TNGTNACNGT NWSNAAYGGN TAYATGTGGG ARYTNAARAC NWSNGARGGN

1381 GGNTGGGGNY TNCAYGAYAT HATHAAYCAR AAYGAYTGGA ARYTNCARGG NATHGTNAAY

1441 GGNATHGAYA TGWSNGARTG GAAYCCNGCN GTNGAYGTNC AYYTNCAYWS NGAYGAYTAY

1501 ACNAAYTAYA CNTTYGARAC NYTNGAYACN GGNAARMGNC ARTGYAARGC NGCNYTNCAR

1561 MGNCARYTNG GNYTNCARGT NMGNGAYGAY GTNCCNYTNA THGGNTTYAT HGGNMGNYTN

1621 GAYCAYCARA ARGGNGTNGA YATHATHGCN GAYGCNATHC AYTGGATHGC NGGNCARGAY

1681 GTNCARYTNG TNATGYTNGG NACNGGNMGN GCNGAYYTNG ARGAYATGYT NMGNMGNTTY

1741 GARWSNGARC AYWSNGAYAA RGTNMGNGCN TGGGTNGGNT TYWSNGTNCC NYTNGCNCAY

1801 MGNATHACNG CNGGNGCNGA YATHYTNYTN ATGCCNWSNM GNTTYGARCC NTGYGGNYTN

1861 AAYCARYTNT AYGCNATGGC NTAYGGNACN GTNCCNGTNG TNCAYGCNGT NGGNGGNYTN

1921 MGNGAYACNG TNGCNCCNTT YGAYCCNTTY AAYGAYACNG GNYTNGGNTG GACNTTYGAY

1981 MGNGCNGARG CNAAYMGNAT GATHGAYGCN YTNWSNCAYT GYYTNACNAC NTAYMGNAAY

2041 TAYAARGARW SNTGGMGNGC NTGYMGNGCN MGNGGNATGG CNGARGAYYT NWSNTGGGAY

2101 CAYGCNGCNG TNYTNTAYGA RGAYGTNYTN GTNAARGCNA ARTAYCARTG GTRRGCNAAY

2161 TRRYTNGCNA CNMGNMGNMG NWSNTGYMGN MGNACNTGGA CNYTNTTYMG NMGNYTNTTY

2221 WSNYTNGCNG CNYTNATGMG NGCNWSNCAY YTNMGNMGNG CNGAYGGNMG NMGNTGGYTN

2281 GCNTAYMGNY TNMGNMGNYT NMGNGCNYTN GGNATHTGGG CNGGNACNAT GATGCCNYTN

2341 GGNACNGGNM GNGGNGTNGT NTRRTAYGAR ACNGAYGGNG AYGGNGAYGA RGCNCAYGGN

2401 ATHTTYCCNY TNATHAAYGG NGARYTNTAY GCNACNYTNA THWSNCCNYT NYTNYTNGTN

2461 TTYATHYTNA TGGCNGCN

CLAIMS

1. A cDNA specifying a soluble starch synthase having the sequences of the inserts in plasmids pSSS6, pSSS10.1 and pSSS6.31 and sequences having sufficient similarity such that when inserted into the genome of an organism which produces starch, the synthesis of starch is altered.

2. The cDNA of the insert of plasmid pSSS6, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 13th June 1994, under the Accession Number 40651.

3. The cDNA of the insert in plasmid pSSS6.31, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Number NCIMB 40679.

4. The cDNA of the insert in plasmid pSSS10.1, deposited under the terms of the Budapest Treaty, with the National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB1 2RY, on 22nd August 1994, under the Accession Number NCIMB 40680.

5. A cDNA, encoding soluble starch synthase which has the sequence SEQ-ID-NO-1, or SEQ-ID-NO-2 or SEQ-ID-NO-3.

6. A transformed plant containing one or more copies of one or more of the said cDNAs claimed in claim 5 in sense or antisense orientation.

7. A method of producing a plant with altered starch synthesising ability comprising stably incorporating into the genome of a recipient plant one or

more than one donor gene specifying soluble starch synthase as claimed in claim 5.

8. A method as claimed in claim 7 in which the recipient plant is of the family Gramineae.

9. A method as claimed in claim 8 in which the recipient plant is of the species Zea mays.

10. Seeds of a plant as claimed in claim 6.

FIGURE 1

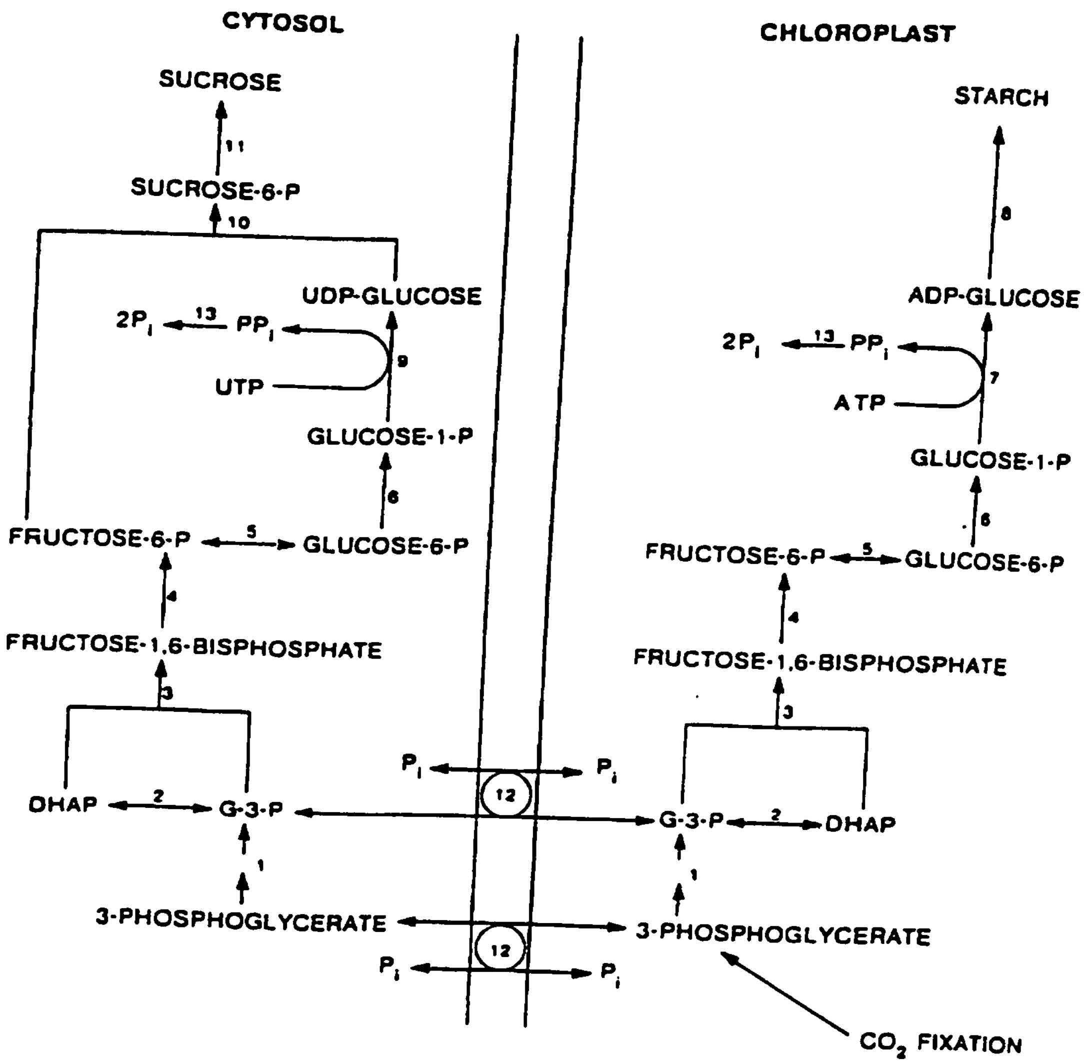
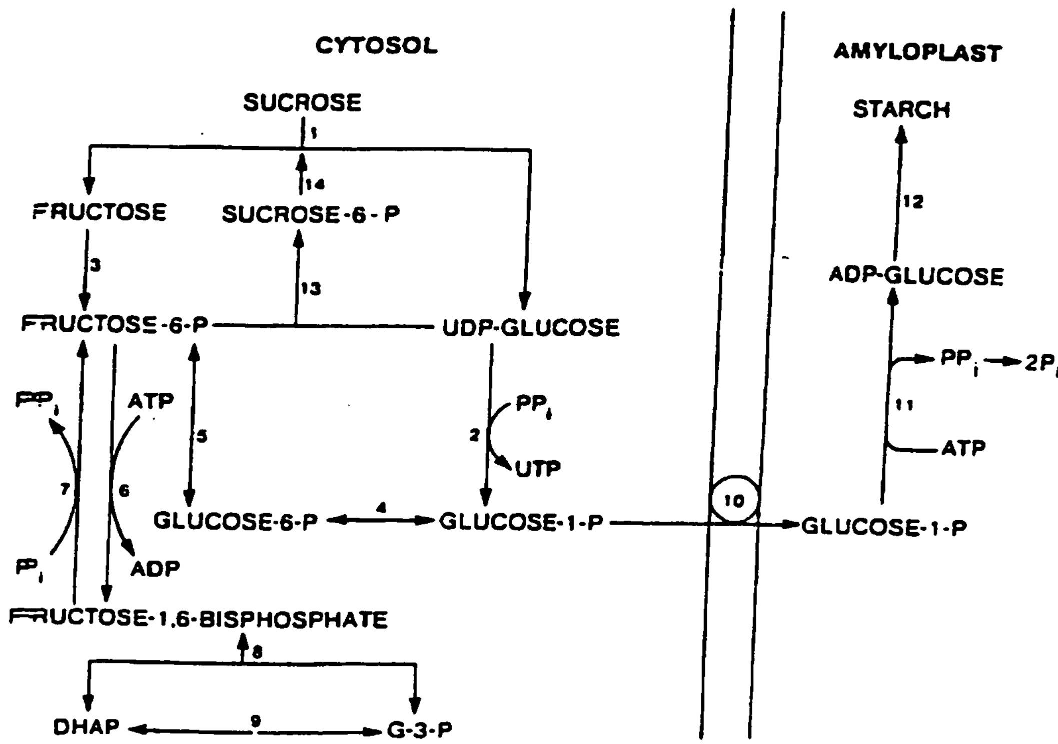

CYTOSOL
CHLOROPLAST
SUCROSE
11
SUCROSE-6-P
10
UDP-GLUCOSE
$2P_i$
13
PP_i
9
UTP
GLUCOSE-1-P
6
FRUCTOSE-6-P
5
GLUCOSE-6-P
4
FRUCTOSE-1,6-BISPHOSPHATE
3
DHAP
2
G-3-P
1
3-PHOSPHOGLYCERATE
P_i
12
STARCH
8
ADP-GLUCOSE
7
ATP
CO$_2$ FIXATION

FIGURE 2


CYTOSOL
AMYLOPLAST
SUCROSE
STARCH
1
14
12
FRUCTOSE
SUCROSE-6-P
ADP-GLUCOSE
3
13
FRUCTOSE-6-P
UDP-GLUCOSE
PPi
2Pi
PPi
ATP
PPi
11
5
2
ATP
UTP
7
6
10
GLUCOSE-6-P
4
GLUCOSE-1-P
GLUCOSE-1-P
Pi
ADP
FRUCTOSE-1,6-BISPHOSPHATE
8
DHAP
9
G-3-P

Applicant's or agent's file reference number SEE 45052/WO	International application No.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page FIVE, line 16 - 23 inclusive.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

NCIMB

Address of depositary institution *(including postal code and country)*

23 St Machar Drive, Aberdeen AB1 2RY

Date of deposit	Accession Number
13.06.94	40651
22.08.94	40679
22.08.94	40680

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Seeds,
Jealotts Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli Sol R containing plasmid pSSS6.31	NCIMB 40679

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 22 August 1994 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd
Address: 23 St Machar Drive
Aberdeen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 23 August 1994

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd Address: 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 23 August 1994

[4] Fill in if the information has been requested and if the results of the test were negative.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

Zeneca Seeds,
Jealotts Hill Research
Station,
Bracknell, Berkshire. RG12 6EY

NAME AND ADDRESS
OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT.
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

Escherichia coli Sol R
containing plasmid pSSS10.1

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 40680

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 22 August 1994 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on . (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd

Address: 23 St Machar Drive
Aberdeen Scotland
UK

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 23 August 1994

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Seeds,
Jealotts Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40680 Date of the deposit or of the transfer:[1] 22 August 1994

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 22 August 1994 [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

Form BP/9 (first page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: NCIMB Ltd Address: 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 23 August 1994

[4] Fill in if the information has been requested and if the results of the test were negative.

Form BP/9 (second and last page)

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Zeneca Limited
Zeneca Seeds
Jealott's Hill Research Station
Bracknell
Berkshire
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

NAME AND ADDRESS
OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:

Escherichia coli Sol R containing plasmid pSSS6

Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:

NCIMB 40651

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 13 June 1994 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: [illegible]
23 [illegible]
Aberdeen
Address: UK [illegible]

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 1? June 1994

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Zeneca Limited
Zeneca Seeds
Jealott's Hill Research Station
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Berkshire
RG12 6EY

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: As above Address:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40651 Date of the deposit or of the transfer:[1] 13 June 1994

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 13 June 1994[2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

[1] Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

Form BP/9 (first page)

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: [illegible] Address: 23 St [illegible] Aberdeen [illegible]	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 14 June 1994

[4] Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02990

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/52 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL., vol. 108, no. 2 (Suppl.), June 1995, page 50, abstract no. 187, C. HARN ET AL.: "Isolation of a starch synthase cDNA clone from maize W64A" XP000651998 see the abstract. & 1995 annual meeting of the Amer. Soc. of Plant Physiol., Charlotte, N. Carolina, USA, July 29th - August 2nd, 1995. ---	1-10
X	WO 94 09144 A (ZENECA LTD.) 28 April 1994 see pages 39-41, Example 4 and Claims. ---	1-10
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 1997	09.05.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Yeats, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 96/02990

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT J., vol. 6, no. 2, 1994, pages 151-159, XP000651922 C. MU ET AL.: "Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm" see the whole document, in particular the summary, Table 1 and the last paragraph of the discussion. ---	1-10
X	PLANT PHYSIOL., vol. 103, 1993, pages 565-573, XP000565731 T. BABA ET AL.: "Identification, cDNA cloning, and gene expression of soluble starch synthase in rice (Oryza sativa L.) immature seeds" cited in the application see the abstract and Figure 5. ---	1,6,7,10
X	J. BIOL. CHEM., vol. 260, 1985, pages 16451-16459, XP000652758 M.D. MARKS ET AL.: "Nucleotide sequence analysis of zein mRNAs from maize endosperm" see abstract and Figure 4, clone cZ19C1. ---	1
A	EP 0 521 621 A (ADVANCED TECHNOLOGIES (CAMBRIDGE) LTD.) 7 January 1993 see the claims. ---	1,6,7
P,X	WO 96 15248 A (INSTITUT FÜR GENBIOLOGISCHE FORSCHUNG BERLIN GMBH) 23 May 1996 see Examples 1-12 and Claims. -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/02990

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9409144 A	28-04-94	AU 2696492 A	09-05-94
		EP 0664835 A	02-08-95
EP 521621 A	07-01-93	CA 2071010 A	13-12-92
		US 5365016 A	15-11-94
WO 9615248 A	23-05-96	DE 4441408 A	15-05-96
		AU 3927995 A	06-06-96

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Published:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MODIFIED GENE-SILENCING RNA AND USES THEREOF

(57) Abstract: Methods and means for efficiently downregulating the expression of any gene of interest in eukaryotic cells and organisms are provided. To this end, the invention provides modified antisense and sense RNA molecules, chimeric genes encoding such modified antisense or sense RNA molecules and eukaryotic organisms such as plants, animals or fungi, yeast or molds comprising the modified antisense and/or sense RNA molecules or the encoding chimeric genes.

WO 03/076619 A1

Modified gene-silencing RNA and uses thereof

Field of the invention

The present invention relates to methods for efficienty downregulating the expression of any gene of interest in eukaryotic cells and organisms. To this end, the invention provides modified antisense and sense RNA molecules, chimeric genes encoding such modified antisense or sense RNA molecules and eukaryotic organisms such as plants, animals or fungi, yeast or molds comprising the modified antisense and/or sense RNA molecules or the encoding chimeric genes.

Background art

Recently, it has been shown that introduction of double stranded RNA (dsRNA) also called interfering RNA (RNAi), or hairpin RNA is an effective trigger for the induction of gene-silencing in a large number of eukaryotic organisms, including animals, fungi or plants.

Both the qualitative level of dsRNA mediated gene silencing (level of gene-silencing within an organism) and the quantitative level (number of organisms showing a significant level of gene-silencing within a population) have proven superior to the more conventional antisense RNA or sense RNA mediated gene silencing methods.

For practical purposes, the production of antisense RNA molecules and chimeric genes encoding such antisense RNA is more straightforward than the production of dsRNA molecules or the encoding genes. Indeed, the chimeric nucleic dsRNA molecules or the encoding genes contain large, more or less perfect inverted repeat structures, and such structures tend to hamper the intact maintenance of these nucleic acids in the intermediate prokaryotic

cloning hosts. The methods and means as hereinafter described to increase the efficiency of antisense-RNA mediated gene silencing provide a solution to this problem as described in the different embodiments and claims.

US 5,190,131 and EP 0 467 349 A1 describe methods and means to regulate or inhibit gene expression in a cell by incorporating into or associating with the genetic material of the cell a non-native nucleic acid sequence. Said sequence is transcribed to produce an mRNA which is complementary to and capable of binding to the mRNA produced by the genetic material of that cell.

EP 0 223 399 A1 describes methods to effect useful somatic changes in plants by causing the transcription in the plant cells of negative RNA strands which are substantially complementary to a target RNA strand. The target RNA strand can be a mRNA transcript created in gene expression, a viral RNA, or other RNA present in the plant cells. The negative RNA strand is complementary to at least a portion of the target RNA strand to inhibit its activity *in vivo*.

EP 0 240 208 describes a method to regulate expression of genes encoded for in plant cell genomes, achieved by integration of a gene under the transcriptional control of a promoter which is functional in the host. In this method, the transcribed strand of DNA is complementary to the strand of DNA that is transcribed from the endogenous gene(s) one wishes to regulate.

WO95/15394 and US 5908779 describe a method and construct for regulating gene expression through inhibition by nuclear antisense RNA in (mouse) cells. The construct comprises a promoter, antisense sequences, and a cis-or trans-ribozyme which generates 3'-ends independently of the polyadenylation machinery and thereby inhibits the transport of the RNA molecule to the cytoplasm.

WO98/05770 discloses antisense RNA with special secondary structures such as $(GC)_n$-palindrome-$(GC)_n$ or $(AT)_n$-palindrome-$(AT)_n$ or $(CG)_n$-palindrome-$(CG)_n$ and the like.

WO 01/12824 discloses methods and means for reducing the phenotypic expression of a nucleic acid of interest in eukaryotic cells, particularly in plant cells, by providing aberrant, preferably unpolyadenylated, target-specific RNA to the nucleus of the host cell. Preferably, the unpolyadenylated target-specifc RNA is provided by transcription of a chimeric gene comprising a promoter, a DNA region encoding the target-specific RNA, a self-splicing ribozyme and a DNA region involved in 3' end formation and polyadenylation.

WO 02/10365 provides a method for gene suppression in eukaryotes by transformation with a recombinant construct containing a promoter, at least one antisense and/or sense nucleotide sequence for the gene(s) to be suppressed, wherein the nucleus-to-cytoplasm transport of the transcription products of the construct is inhibited. In one embodiment, nucleus-to-cytoplasm transport is inhibited by the absence of a normal 3' UTR. The construct can optionally include at least one self-cleaving ribozyme. The construct can also optionally include sense and/or antisense sequences to multiple genes that are to be simultaneously down-regulated using a single promoter. Also disclosed are vectors, plants, animals, seeds, gametes, and embryos containing the recombinant constructs.

Zhao et al., J. Gen. Virology, 82, 1491-1497 (2001) described the use of a vector based on Potato Virus X in a whole plant assay to demonstrate nuclear targeting of Potato spindle tuber viroid (PSTVd).

WO 02/00894 relates to gene silencing methods wherein the nucleic acid constructs comprise within the transcribed region a DNA sequence which consists of a stretch of T bases in the transcribed strand.

WO 02/00904 relates to gene silencing methods wherein nucleic acid constructs comprise (or encode) homology to at least one target mRNA expressed by a host, and in the proximity thereto, two complementary RNA regions which are unrelated to any endogenous RNA in the host.

Summary of the invention

In one embodiment of the invention a method for down regulating the expression of a target gene in cells of a eukaryotic organisms is provided, comprising the steps of

a) providing the cells of the eukaryotic organism with a chimeric RNA molecule wherein the chimeric RNA molecule comprises

   i) one target-gene specific region or multiple target-gene specific regions comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequence identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene; operably linked to

   ii) a largely double stranded RNA region comprising a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type such as Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid or a the largely double stranded RNA region or a largely double stranded RNA region comprising at least 35 repeats of the trinucleotides CUG, CAG, GAC or GUC such as between 44 and 2000 repeats of these trinucleotide ; and

b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

The chimeric RNA molecule may comprise an intron sequence. The viroids may have a genomic nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8. The eukaryotic organism may be a plant including a plant selected from *Arabidopsis*, alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, wheat, asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucmber, eggplant, lettuce, onion, oilseed rape, pepper, potato, pumpkin, radish, spinach, squash, tomato, zucchini, almond, apple, apricot, banana, blackberry, b lueberry, cacao, cherry, coconut, cranberry, date, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut and watermelon. The eukaryotic organism may also be a fungus, yeast or mold or an animal such as a human, mammal, fish, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, guinea pig, rabbit, primate, nematode, shellfish, prawn, crab, lobster, insect, fruit fly, Coleapteran insect, Dipteran insect, Lepidopteran insect and Homeopteran insect.

It is an object of the invention to provide a chimeric RNA molecule for down-regulating the expression of a target gene in a cell of a eukaryotic organisms, comprising one target-gene specific region or multiple target-gene specific regions a target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene; operably linked to a largely double stranded RNA region comprising a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type such as Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian

grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid or a the largely double stranded RNA region or a largely double stranded RNA region comprising at least 35 repeats of the trinucleotide CUG, CAG, GAC OR GUC such as between 44 and 2000 repeats of the trinucleotide CUG, CAG, GAC OR GUC wherein the chimeric RNA molecule, when provided to cells of the eukaryotic organism down-regulates the expression of the target gene.

It is another object of the invention to provide a chimeric DNA molecule for reduction of the expression of a target gene in a cell of a eukaryotic organism, comprising

a) a promoter or promoter region capable of being recognized by RNA polymerases in the cells of the eukaryotic organism ; operably linked to
b) a DNA region, which when transcribed yields an RNA molecule, the RNA molecule comprising
   i) one target-gene specific region or multiple target-gene specific regions comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene; operably linked to
   ii) a largely double stranded RNA region comprising a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type such as Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid or a the largely double stranded RNA region or a largely double stranded RNA region comprising at least 35 repeats of the trinucleotide CUG, CAG, GAC OR GUC such as between 44

and 2000 repeats of the trinucleotide CUG, CAG, GAC OR GUC ; and optionally

iii) further comprising a transcription termination and polyadenylation signal operably linked to the DNA region encoding the RNA molecule.

wherein the chimeric DNA molecule, when provided to cells of the eukaryotic organism reduces the expression of the target gene.

Depending on the eukaryotic host organism, the promoter or promoter region may a promoter which functions in animals, a promoter which functions in yeast, fungi or molds or a plant-expressible promoter. The promoter may also be a promoter or promoter region recognized by a single subunit bacteriophage RNA polymerase.

The invention also provides cells from a eukaryotic organism comprising a chimeric DNA or RNA molecules according to the invention, as well as non-human eukaryotic organisms, comprising in their cells a chimeric DNA or RNA molecule according to the invention.

It is yet another object of the invention to provide the use of a chimeric RNA or DNA molecule according to the invention for reduction of the expression of a target gene in a cell of a eukaryotic organism.

The invention also provides a method for making a transgenic eukaryotic organism wherein expression of a target gene in cells of the organism is reduced, the method comprising the steps of :

a) providing a chimeric DNA molecule according to the invention to a cell or cells of the organism to make a transgenic cell or cells; and
b) growing or regenerating a transgenic eukaryotic organism from the transgenic cell or cells.

The invention also provides a method for down regulating the expression of a target gene in cells of a eukaryotic organisms, comprising the steps of

a) providing the cells of the eukaryotic organism with a first and second chimeric RNA molecule, wherein
   i) the first chimeric RNA molecule comprises an antisense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ;
   ii) the second chimeric RNA molecule comprises a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the complement of the first chimeric RNA molecule ;
   iii) the first and second chimeric RNA are capable of basepairing at least between the 19 consecutive nucleotides of the first chimeric RNA and the 19 consecutive nucleotides of the second chimeric RNA ; and
   iv) wherein either the first or the second chimeric RNA molecule comprises a largely double stranded RNA region operably linked to the antisense target-specific RNA region or to the sense target-specific RNA region ; and
b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

Both the first and second chimeric RNA molecule may comprise a largely double stranded region.

It is another object of the invention to provide a cell from a eukaryotic organism, (as well as non-human eukaryotic organisms comprising such cells), comprising a first and second chimeric RNA molecule,

i) the first chimeric RNA molecule comprising an antisense target-gene specific RNA region comprising a nucleotide sequence of at least

about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ;

ii) the second chimeric RNA molecule comprising a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the complement of the first chimeric RNA molecule ;

iii) the first and second chimeric RNA being capable of basepairing at least between the 19 consecutive nucleotides of the first chimeric RNA and the 19 consecutive nucleotides of the second chimeric RNA ; and

wherein either the first or the second chimeric RNA molecule comprises a largely double stranded RNA region operably linked to the antisense target-specific RNA region or to the sense target-specific RNA region.

The invention further provides chimeric sense RNA molecules or chimeric DNA molecules encoding such chimeric sense RNA molecules for reduction of expression of a target gene in a cell of a eukaryotic organism in cooperation with a chimeric antisense RNA molecule, wherein the chimeric sense RNA molecule comprises a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the nucleotide of said target gene ; operably linked to a largely double stranded RNA region.

Brief description of the figures.

Figure 1 : Schematic representation of the secondary structure predicted using Mfold software for different viroids of the PSTVd-type. A. Potato spindle tuber viroid ; B. Australian grapevine viroid ; C. Coconut tinangaja viroid ; D. Tomato planta macho viroid ; E. Hop latent viroid of thermomutant T229 ; F. Tomato apical stunt viroid.

Figure 2 : schematic representation of the various chimeric gene constructs used in the examples 1 to 3 of this application. 35S-P : CaMV 35S promoter ; Pdk intron : *Flaveria trinervia* pyruvate orthophosphate dikinase 2 intron 2; cEIN2 : cDNA copy of the EIN2 gene from *Arabidopsis* (gene required for sensitivity to ehylene ; Alonso et al. 1999 Science 284, 2148-2152) the orientation of this region with respect to the promoter is indicated by the arrow; gEIN2 : genomic copy of the EIN2 gene from *Arabidopsis* ; PSTVd : cDNA copy of the genome of potato spindle tuber viroid ; PSTVd* : partial sequence from PSTVd from nucleotide 16 to nucleotide 355, cloned in inverse orientation with regard to the intact copy of PSTVd ; OCS 3' : 3' region of the octopine synthase gene from *Agrobacterium tumefaciens.*

Figure 3 : Phenotype of EIN2-silenced plants when germinating on 1-aminocyclopropane-1-carboxylic acid (ACC). A. In the dark ; B . under light conditions. Wt : wild-type plants.

Figure 4 : schematic representation of the various chimeric gene constructs used in Example 4. CMV promoter: cytomegolovirus promoter ; SV40 poly(A) : transcription termination and polyadenylation region from SV40 ; PSTVd : potato spindle tuber viroid sequence ; CUGrep : sequence comprising 60 repeats of the CUG sequence ; humGFP : humanized green fluorescent protein coding region (adapted to the codon usage of human genes ; the orientation of this region with respect to the promoter is indicated by the arrow);

Fig 5 : Schematic representation of the predicted secondary structure of pSTVd in pMBW491 (A ;adopting almost the wild type configuration) and in pMBW489, where a 10 nucleotide deletion results in a structure different from the wild type configuration.

Detailed description of the different embodiments.

The currently described method and means for obtaining enhanced antisense RNA -mediated down regulation of gene expression are based upon the unexpected observation that operably linking the target gene-specific RNA sequence to a largely double stranded RNA region, such as an RNA region comprising the nucleotide sequence of a Potato spindle tuber viroid genome, which in turn comprises a nuclear localization signal for the RNA in which it is embedded, when introduced into cells of a host organism, such as a plant cell, increased both the number of lines wherein gene expression of the target gene was down-regulated, as well as the number of lines wherein gene expression of the target gene was significantly downregulated or even abolished.

Thus, in one embodiment of the invention, a method is provided for down regulating the expression of a target gene in cells of a eukaryotic organisms, comprising the steps of

a) providing the cells of the eukaryotic organism with a chimeric RNA molecule wherein the RNA molecule comprises
    i) a target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene (the « antisense RNA ») ; operably linked to
    ii) a largely double stranded RNA region ; and
b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

« Chimeric gene » or « chimeric nucleic acid » as used herein, refers any gene or any nucleic acid, which is not normally found in a particular eukaryotic species or, alternatively, any gene in which the promoter is not associated in

nature with part or all of the transcribed DNA region or with at least one other regulatory region of the gene.

As used herein, « antisense RNA » refers to RNA molecules which comprise a nucleotide sequence that is largely complementary to part of the nucleotide sequence of the biologically active RNA, usually but not exclusively mRNA, which is transcribed from the target gene.

The expression « target gene » is used herein to refer to any nucleic acid which is present in the eukaryotic cell and that is transcribed into a biologically active RNA. The target gene may be an endogenous gene, it may be a transgene that was introduced through human intervention in the ancestors of the eukaryotic cell, or it may be a gene introduced into the genome of the cell by infectious organisms such as e.g. *Agrobacterium* strains or retroviruses. The target gene may also be of viral origin. Furthermore, the stretch of at least 19 nucleotides may be selected from the promoter region, the 5'UTR, the coding region, or the 3'UTR.

"Gene expression" or "expression of a nucleic acid" is used herein to refer to the process wherein a gene or nucleic acid, when introduced in a suitable host cell, can be transcribed (or replicated) to yield an RNA, and/or translated to yield a polypeptide or protein in that host cell.

As used herein, "downregulation of gene expression" refers to the comparison of the expression of the target gene or nucleic acid of interest in the eukaryotic cell in the presence of the RNA or chimeric genes of the invention, to the expression of target gene or the nucleic acid of interest in the absence of the RNA or chimeric genes of the invention. The expression of the target gene in the presence of the chimeric RNA of the invention should thus be lower than the expression in absence thereof, such as be only about 50% or 25% or about 10% or about 5% of the phenotypic expression in absence of the chimeric RNA. For a number of applications, the expression should be completely

inhibited for all practical purposes by the presence of the chimeric RNA or the chimeric gene encoding such an RNA.

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

It will thus be clear that the minimum nucleotide sequence of the antisense RNA of about 19 nt of the target-gene specific RNA region may be comprised within a larger RNA molecule, varying in size from 19 nt to a length equal to the size of the target gene with a varying overall degree of sequence identity.

For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e., a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970) The computer-assisted sequence alignment above, can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madision, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3. Sequences are indicated as "essentially similar" when such sequence have a sequence identity of at least about 75%, particularly at least about 80 %, more

particularly at least about 85%, quite particularly about 90%, especially about 95%, more especially about 100%, quite especially are identical. It is clear than when RNA sequences are the to be essentially similar or have a certain degree of sequence identity with DNA sequences, thymine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence. Thus when it is stated in this application that a sequence of 19 consecutive nucleotides has a 94% sequence identity to a sequence of 19 nucleotides, this means that at least 18 of the 19 nucleotides of the first sequence are identical to 18 of the 19 nucleotides of the second sequence.

The mentioned antisense nucleotide regions may thus be about 21nt, 50 nt, 100nt, 200 nt, 300nt, 500nt, 1000 nt, 2000 nt or even about 5000 nt or larger in length, each having an overall sequence identity of about 40 % or 50% or 60 % or 70% or 80% or 90 % or 100%. The longer the sequence, the less stringent the requirement for the overall sequence identity is.

Furthermore, multiple sequences with sequence identity to the complement of the nucleotide sequence of a target gene (multiple target-gene specific RNA regions) may be present within one RNA molecule. Also, multiple sequences with sequence identity to the complement of the nucleotide sequences of several target genes may be present within one RNA molecule.

« Target-gene specific » is not to be interpreted in the sense that the chimeric nucleic acids according to the invention can only be used for down-regulation of that specific target gene. Indeed, when sufficient homology exists between the target gene specific RNA region and another gene, or when other genes share the same stretch of 19 nucleotides (such as genes belonging to a so-called gene-family) expression of those other genes may also be down-regulated.

As used herein, a « largely double stranded RNA region » refers to an RNA molecule which is capable of folding into a rod-like structure by internal base-

pairing and wherein the resulting rod-like structure does not comprise any stretch of 19 consecutive nucleotides having 94% sequence identity to the complement of another stretch of 19 other consecutive nucleotides within that RNA molecule, which are capable of forming a double stranded region when the RNA molecule folds into a rod-like structure. In other words, the largely double stranded RNA region upon folding does not contain a double stranded RNA regions of at least 19 bp with at most one mismatch in those 19 bp, at least not in the energicatically most favourable rod-like confirmation. Non-limiting examples of such structures are represented in figure 1.

Although not intending to limit the invention to a specific mode of action, it is thought that such largely double stranded RNA regions are involved in the nuclear localization of the antisense RNA molecules with which they are associated. As a consequence thereof, the concentration of the antisense RNA molecules in the nucleus may be increased, allowing a more efficient formation of the formation of sequence specific dsRNA formation by base pairing with the sense RNA corresponding to the antisense RNA.

As used herein, the term « Capable of folding into a rod-like structure » with regard to an RNA molecule refers to a secondary structure which the RNA molecule will preferably adapt by internal basepairing and which has the overall appearance of a long rod. The rod-like structure may comprise branches or bulges (where non-matching nucleotides bulge out from the overall structure) and may be part of a larger secondary structure (which may or may not be rod-like). Examples of RNA molecules capable of folding into a rod-like structure are represented in Figure 1.

The specific secondary structure adapted will be determined by the free energy of the RNA molecule, and can be predicted for different situations using appropriate software such as FOLDRNA (Zuker and Stiegler, 1981) or the

MFOLD structure prediction package of GCG (Genetics Computing Group; Zuker 1989, Science 244, 48-52).

In one embodiment of the invention, the largely double stranded RNA region operably linked to the antisense RNA molecule is a nuclear localization signal from a viroid of the PSTVd type, such as PSTVd (Potato spindle tuber viroid), capable of replicating in the nucleus of the host cell or host plant cell.

In one embodiment of the invention, the largely double stranded RNA region comprises the full length sequence of PSTVd strain RG1, which can conveniently be obtained by amplification from a cDNA copy of the RNA genome of the viroid using oligonucleotide primers with the nucleotide sequence

5'-cgcagatctcggaactaaactcgtggttc-3' [SEQ ID N°1] and

5'gcgagatctaggaaccaactgcggttc-3'[SEQ ID N°2]),

such as the nucleotide sequence represented in SEQ ID N°3.

It is understood that for incorporation in an RNA molecule, an additional step is required to convert the DNA molecule in the corresponding RNA molecule. Such a conversion may be achieved by transcription, e.g. in vitro transcription using a single subunit bacteriophage RNA polymerase.

It is also clear than when RNA sequences are said to be represented in an entry in the Sequence Listing or to be essentially similar or have a certain degree of sequence identity with DNA sequences represented in the Sequence Listing, reference is made to RNA sequences corresponding to the sequences in the entries, except that thymine (T) in the DNA sequence is replaced by uracil (U) in the RNA sequence. Whether the reference is to RNA or DNA sequence will be immediately apparent by the context.

Similar largely double stranded RNA structures are also found within the genomes of other nuclear-replicating viroids of the PSTVd type (or group B according to the classification by Bussière et al. 1996) and these RNA sequences may be used to similar effect. Other nuclear-replicating viroids of the PSTVd group include Citrus viroid species III, Citrus viroid species IV, Coleus viroid, Hop latent viroid (SEQ ID N° 7), Australian grapevine viroid (SEQ ID N° 4), Tomato planta macho viroid (SEQ ID N° 6), Coconut tinangaja viroid (SEQ ID N° 5), Tomato apical stunt viroid (SEQ ID N° 8), Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid or Citrus bent leaf viroid. These viroids are also characterized by the absence of self-splicing activity which becomes apparent by the absence of catalytic motifs such as the hammerhead motif (Busière et al. Nuc. Acids Res. 24, 1793-1798, 1996). The longest stretch of perfect dsRNA structures among all the PSTVd-type of viroids is 11 base pairs in size. The mismatches are usually quite evenly distributed.

Nucleotide sequences for these viroids have been compiled in a database accesible via the worldwide web (http://www.callisto.si.usherb.ca/~jpperra or http://nt.ars-grin.gov/subviral/) and include the following :

Potato spindle tuber viroid (PSTVd) [PSTVd.1 (Accession numbers: J02287(gb), M16826(gb), V01465(embl); 333351(gi), 333352(gi) and 62283(gi)); PSTVd.2 (Accession numbers: M38345(gb), 333354(gi)); PSTVd.3 (Accession numbers: M36163(gb), 333356(gi)); PSTVd.4 (Accession numbers: M14814(gb), 333357(gi)); PSTVd.5 (strain: S.commersonii) (Accession numbers: M25199(gb), 333355(gi)); PSTVd.6 (strain: tomato cv. Rutgers,isolate: KF440-2) (Accession numbers: X58388(embl), 61366(gi)); PSTVd.7 (mild strain KF6-M) (Accession number: M88681(gb), 333358(gi)); PSTVd.8 (strain Burdock) (Accession numbers: M88678(gb), 333360(gi)); PSTVd.9 (strain Wisconsin (WB)) (Accession numbers: M88677(gb), 333359(gi)); PSTVd.10 (strain PSTVd-N(Naaldwijk)) (Accession numbers:

X17268(embl), 60649(gi)) ;PSTVd.11 (mild strain variant A, WA-M isolate) (Accession numbers: X52036(embl), 61365(gi)); PSTVd.12 (mild strain, F-M isolate) (Accession numbers: X52037(embl), 61367(gi)); PSTVd.13 (intermediate-severe strain, F-IS isolate) (Accession numbers: X52039(embl), 61369(gi)); PSTVd.14 (severe-lethal strain, F-SL isolate) (Accession numbers: X52038(embl), 61368(gi)); PSTVd.15 (intermediate-severe strain, F88-IS isolate) as published in Herold,T et al., *Plant Mol. Biol.* **19**, 329-333 (1992); PSTVd.16 (variant F88 or S88)(Accession numbers: X52040(embl), 61370(gi)); PSTVd.17 (individual isolate kf 5) (Accession numbers: M93685(gb), 333353(gi)); PSTVd.18 (isolate KF5) (Accession numbers: S54933(gb), 265593(gi)); PSTVd.19 (strain S-XII, variety s27) (Accession numbers: X76845(embl), 639994(gi)); PSTVd.20 (strain S-XIII, variety s23) (Accession numbers: X76846(embl), 639993(gi)); PSTVd.21 (strain M(mild)) (Accession numbers: X76844(embl), 639992(gi)); PSTVd.22 (strain I-818, variety I4) (Accession numbers: X76848(embl), 639991(gi)); PSTVd.23 (strain I-818, variety I3) (Accession numbers: X76847(embl), 639990(gi)); PSTVd.24 (strain PSTVd-341) (Accession numbers: Z34272(embl), 499191(gi)); PSTVd.25 (strain QF B) (Accession numbers: U23060(gb), 755586(gi)) PSTVd.26 (strain QF A) (Accession numbers: U23059(gb), 755585(gi)); PSTVd.27 (strain RG 1) (Accession numbers: U23058(gb), 755584(gi)); PSTVd.28 (Accession numbers: U51895(gb), 1272375(gi)); PSTVd.29(Potato spindle tuber viroid) (Accession numbers: X97387(embl), 1769438(gi)); PSTVd.30 (strain S27-VI-24) (Accession numbers: Y09382(emb), 2154945(gi)); PSTVd.31 (strain S27-VI-19) (Accession numbers: Y09383(emb), 2154944(gi)); PSTVd.32 (strain SXIII) (Accession numbers: Y08852(emb), 2154943(gi)); PSTVd.33 (strain S27-I-8) (Accession numbers: Y09381(emb), 2154942(gi)); PSTVd.34 (strain PSTV M-VI-15) (Accession numbers: Y09577(emb), 2154941(gi)); PSTVd.35 (strain PSTV M-I-40) (Accession numbers: Y09576(emb), 2154940(gi)); PSTVd.36 (strain PSTV M-I-17) (Accession numbers: Y09575(emb), 2154939(gi)); PSTVd.37 (strain PSTV M-I-10) (Accession numbers: Y09574(emb), 2154938(gi)); PSTVd.38 (variant I4-I-42) (Accession numbers:

Y09889(emb), 2154937(gi)); PSTVd.39 (variant PSTVd I2-VI-27) (Accession numbers: Y09888(emb), 2154936(gi)); PSTVd.40 (variant PSTVd I2-VI-25) (Accession numbers: Y09887(emb), 2154935(gi)); PSTVd.41 (variant PSTVd I2-VI-16) (Accession numbers: Y09886(emb), 2154934(gi)); PSTVd.42 (variant PSTVd I4-I-10) (Accession numbers: Y09890(emb), 2154933(gi)); PSTVd.43 (variant PSTVd I2-I-14) (Accession numbers: Y09891(emb), 2154932(gi)) ; PSTVd.44 (isolate KF7) (Accession numbers: AJ007489(emb), 3367737(gi)); PSTVd.45 (Accession numbers: AF369530, 14133876(gi)] ;

Group III citrus viroid (CVd-III) [CVd-III.1 (Accession numbers: S76452(gb), 913161(gi)); CVd-III.2 (Australia New South Wales isolate) (Accession numbers: S75465(gb) and S76454(gb), 914078(gi) and 913162(gi)); CVd-III.3 (Accession numbers: AF123879, GI:7105753); CVd-III.4 (Accession numbers: AF123878, GI:7105752) CVd-III.5 (Accession numbers: AF123877, GI:7105751); CVd-III.6 (Accession numbers: AF123876, GI:7105750); CVd-III.7 (Accession numbers: AF123875, GI:7105749); CVd-III.8 (Accession numbers: AF123874, GI:7105748); CVd-III.9 (Accession numbers: AF123873, GI:7105747); CVd-III.10 (Accession numbers: AF123872, GI:7105746); CVd-III.11 (Accession numbers: AF123871, GI:7105745); CVd-III.12 (Accession numbers: AF123870, GI:7105744); CVd-III.13 (Accession numbers: AF123869, GI:7105743); CVd-III.14 (Accession numbers: AF123868, GI:7105742); CVd-III.15 (Accession numbers: AF123867, GI:7105741); CVd-III.16 (Accession numbers: AF123866, GI:7105740); CVd-III.17 (Accession numbers: AF123865, GI:7105739); CVd-III.18 (Accession numbers: AF123864, GI:7105738) CVd-III.19 (Accession numbers: AF123863, GI:7105737); CVd-III.20 (Accession numbers: AF123860, GI:7105736); CVd-III.21 (Accession numbers: AF123859, GI:7105735); CVd-III.22 (Accession numbers: AF123858, GI:7105734); CVd-III.23 (Accession numbers: AB054619, GI:13537479); CVd-III.24 (Accession numbers: AB054620, GI:13537480); CVd-III.25 (Accession numbers: AB054621, GI:13537481); CVd-III.26 (Accession numbers: AB054622, GI:13537482); CVd-III.27 (Accession numbers: AB054623, GI:13537483); CVd-III.28 (Accession numbers: AB054624, GI:13537484); CVd-III.29

(Accession numbers: AB054625, GI:13537485); CVd-III.30 (Accession numbers: AB054626, GI:13537486); CVd-III.31 (Accession numbers: AB054627, GI:13537487); CVd-III.32 (Accession numbers: AB054628, GI:13537488); CVd-III.33 (Accession numbers: AB054629, GI:13537489); CVd-III.34 (Accession numbers: AB054630, GI:13537490); CVd-III.35 (Accession numbers: AB054631, GI:13537491); CVd-III.36 (Accession numbers: AB054632, GI:13537492); CVd-III.37 (Accession numbers: AF416552, GI:15811643); CVd-III.38 (Accession numbers: AF416553, GI:15811644); CVd-III.39 (Accession numbers: AF416374, GI:15788948); CVd-III.40 (Accession number: AF434680)];

Citrus viroid IV (CVdIV) [CVdIV.1 (Accession numbers: X14638(embl), 59042(gi))]

Coleus blumei-1 viroid (CbVd-1) [CbVd.1 (Coleus blumei viroid 1 (CbVd 1),strain cultivar Bienvenue, german isolate) (Accession numbers: X52960(embl), 58844(gi)); CbVd.2 (Coleus yellow viroid (CYVd), Brazilian isolate) (Accession numbers: X69293(embl), 59053(gi)); CbVd.3 (Coleus blumei viroid 1-RG stem-loop RNA.) (Accession numbers: X95291(embl), 1770104(gi)); CbVd.4 (Coleus blumei viroid 1-RL RNA) (Accession numbers: X95366(embl), 1770106(gi))]

Coleus blumei-2 viroid (CbVd-2) [CbVd.1 (Coleus blumei viroid 2-RL RNA) (Accession numbers: X95365(embl), 1770107(gi)); CbVd.2 (Coleus blumei viroid CbVd 4-1 RNA) (Accession mumbers: X97202(embl), 1770109(gi))]

Coleus blumei-3 viroid (CbVd-3) [CbVd.1 (Coleus blumei viroid 3-RL) (Accession mumbers: X95364(embl), 1770108(gi)); CbVd.2 (Coleus blumei viroid 8 from the Coleus blumei cultivar 'Fairway Ruby') (Accession numbers: X57294(embl),780766(gi)); CbVd.3 (Coleus blumei viroid 3-FR stem-loop RNA, from the Coleus blumei cultivar 'Fairway Ruby') (Accession numbers: X95290(embl), 1770105(gi))]

Hop latent viroid (HLVd)

[HLVd.1 (Accession numbers: X07397(embl), 60259(gi)); HLVd.2 ('thermomutant' T15) (Accession numbers: AJ290404(gb), 13872743(gi));

HLVd.3 ('thermomutant' T40)(Accession numbers: AJ290405.1(gb), 13872744(gi)); HLVd.4 ('thermomutant' T50)(Accession numbers: AJ290406(gb), 13872745(gi)); HLVd.5 ('thermomutant' T59)(Accession numbers: AJ290406(gb), 13872746(gi)); HLVd.6 ('thermomutant' T61) (Accession numbers: AJ290408(gb) 13872747(gi)); HLVd.7 ('thermomutant' T75)(Accession numbers: AJ290409(gb), 13872748(gi)); HLVd.8 ('thermomutant' T92) (Accession numbers: AJ290410(gb), 13872749(gi)); HLVd.9 ('thermomutant' T218) (Accession numbers: AJ290411(gb), 13872750(gi)); HLVd.10 ('thermomutant' T229)(Accession numbers: AJ290412(gb), 13872751(gi))]

Australian grapevine viroid (AGVd) [AGVd.1 (Accession numbers: X17101(embl), 58574(gi))]

Tomato planta macho viroid (TPMVd) [TPMVd.1 (Accession numbers: K00817(gb))]

Coconut tinangaja viroid (CTiVd) [CTiVd.1 (Accession numbers: M20731(gb), 323414(gi))]

Tomato apical stunt viroid (TASVd) [TASVd.1 (Accession numbers K00818(gb), 335155(gi)); TASVd.2 (strain: indonesian) (Accession numbers: X06390(embl), 60650(gi)); TASVd.3(Tomato apical stunt viroid-S stem-loop RNA.) (Accession numbers: X95293(embl), 1771788(gi))]

Cadang-cadang coconut viroid (CCCVd) [CCCVd.1 (isolate baao 54, ccRNA 1 fast) (Accession numbers: J02049(gb), 323275(gi)); CCCVd.2 (isolate baao 54, ccRNA 1 fast) (Accession numbers: J02050(gb), 323276(gi)); CCCVd.3 (isolate baao 54, ccRNA 1 slow) (Accession numbers: J02051(gb), 323277(gi)); CCCVd.4 (isolates Ligao 14B, 620C, 191D and T1, ccRNA 1 fast) (Haseloff et al. *Nature* **299**, 316-321 (1982)) CCCVd.5 (isolates Ligao T1, ccRNA 1 slow) (Haseloff et al. *Nature* **299**, 316-321 (1982)); CCCVd.6 (isolates Ligao 14B, ccRNA 1 slow) (Haseloff et al. *Nature* **299**, 316-321 (1982)); CCCVd.7 (isolate San Nasciso, ccRNA 1 slow) (Haseloff et al. *Nature* **299**, 316-321 (1982))]

Citrus exocortis viroid (CEVd) [CEVd.1 (cev from gynura) (Accession numbers: J02053(gb), 323302(gi)); CEVd.2 (strain A) (Accession numbers:

M34917(gb), 323305(gi)); CEVd.3 (strain de25)(Accession numbers: K00964(gb), 323303(gi)); CEVd.4 (strain de26) (Accession numbers: K00965(gb), 323304(gi)); CEVd.5 (CEV-JB) (Accession numbers: M30870(gb), 484119(gi)); CEVd.6 (CEV-JA) (Accession numbers: M30869(gb), 484118(gi)); CEVd.7 (Accession numbers: M30871(gb), 484117(gi)); CEVd.8 (CEV-A)(Accession numbers: M30868(gb), 484116(gi)); CEVd.9 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)) CEVd.10 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.11 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.12 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.13 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.14 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.15 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.16 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.17 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.18 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.19 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.20 (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.21 (cev-j classe B) (Visvader,J.E. and Symons,R.H. *Nucleic Acids Res.* **13**, 2907-2920 (1985)); CEVd.22 (Grapevine viroid (GV)) (Accession numbers: Y00328(embl), 60645(gi)); CEVd.23 (CEVd-t) (Accession numbers: X53716(embl), 433503(gi)); CEVd.24 (CEVcls, isolate tomato hybrid callus) (Accession numbers: S67446(gb), 141247(gi)); CEVd.25 (CEV D-92) (Accession numbers: S67442(gb), 141248(gi)); CEVd.26 (CEVt, isolate tomato hybrid) (Accession numbers: S67441(gb), 141246(gi)); CEVd.27 (CEVt, isolate tomato)(Accession numbers: S67440(gb), 141245(gi)); CEVd.28 (CEVg, isolate Gynura) (Accession numbers: S67438(gb), 141244(gi)); CEVd.29 (CEVc, isolate citron)(Accession numbers: S67437(gb), 141243(gi)); CEVd.30 (strain CEVd-225) (Accession numbers: U21126(gb), 710360(gi)); CEVd.31 (isolate broad bean, *Vicia faba* L.) (Accession numbers:

S79831(gb),1181910(gi)); CEVd.32 (variant obtain after inoculation tomato with cevd.31) (Fagoaga et al. *J. Gen. Virol.* **76**, 2271-2277 (1995));

CEVd.33 (Fagoaga et al. *J. Gen. Virol.* **76**, 2271-2277 (1995)); CEVd.34 (Accession numbers: AF298177, 15419885(gi)); CEVd.35 (Accession numbers: AF298178, 15419886(gi)); CEVd.36 (Accession: AF428058)(Citrus exocortis viroid isolate 205-E-1 Uy, complete genome.); CEVd.37 (Accession: AF428059) (Citrus exocortis viroid isolate 205-E-2 Uy, complete genome.); CEVd.38 (Accession: AF428060) (Citrus exocortis viroid isolate 205-E-5 Uy, complete genome.); CEVd.39 (Accession: AF428061) (Citrus exocortis viroid isolate 205-E-7 Uy, complete genome.); CEVd.40 (Accession: AF428062) (Citrus exocortis viroid isolate 54-E-1 Uy, complete genome.); CEVd.41 (Accession: AF428063) (Citrus exocortis viroid isolate 54-E-3 Uy, complete genome.); CEVd.42 (Accession: AF428064) (Citrus exocortis viroid isolate 54-E-18 Uy, complete genome.); CEVd.43 (Accession: AF434678) (Citrus exocortis viroid, complete genome.)]

Columnea latent viroid (CLVd) [CLVd.1 (Accession numbers: X15663(embl), 58886(gi)); CLVd.2 (CLVd-N, individual isolate Nematanthus) (Accession numbers: M93686(gb), 323335(gi)); CLVd.3(Columnea latent viroid-B stem-loop RNA) (Accession numbers: X95292(embl), 1770174(gi))]

Citrus bent leaf viroid (CBLVd) [CBLVd.1 (CVd-Ib) (Accession numbers: M74065(gb), 323413(gi)); CBLVd.2 (strain CBLVd-225) (Accession numbers: U21125(gb), 710359(gi)); CBLVd.3 (viroid Ia genomic RNA, isolate: Jp) (Accession numbers: AB006734(dbj), 2815403(gi)); CBLVd.4 (viroid Ib genomic RNA, isolate: P2) (Accession numbers: AB006735(dbj), 2815401(gi)); CBLVd.5 (viroid Ia genomic RNA) (Accession numbers: AB006736(dbj), 2815402(gi)); CBLVd.6 (Citrus Viroid Ia clone 17) (Accession numbers: AF040721(gb), 3273626(gi)); CBLVd.7 (Citrus Viroid Ia clone 18) (Accession numbers: AF040722(gb), 3273627(gi)); CBLVd.8 (Citrus bent leaf viroid isolate 201-1-1 Uy, complete genome.) (Accession: AF428052); CBLVd.9 (Citrus bent leaf viroid isolate 201-1-2 Uy, complete genome.) (Accession: AF428053); CBLVd.10 (Citrus bent leaf viroid isolate 201-1-5 Uy, complete genome.)

(Accession: AF428054); CBLVd.11 (Citrus bent leaf viroid isolate 205-1-1 Uy, complete genome.) (Accession: AF428055); CBLVd.12 (Citrus bent leaf viroid isolate 205-1-3 Uy, complete genome.) (Accession: AF428056); CBLVd.13 (Citrus bent leaf viroid isolate 205-1-4 Uy, complete genome.) (Accession: AF428057)]

Hop stunt viroid (HSVd) [HSVd.h1 (Japanese type strain) (Accession numbers: X00009(embl), 60684(gi)); HSVd.h2 (Japanese strain, variant 2) (Lee et al. *Nucleic Acids Res.* **16**, 8708-8708 (1988)); HSVd.h3 (Korean strain) (Accession numbers: X12537(embl), 60421(gi)); HSVd.g1 (Grapevine viroid (GVVd), isolate SHV-g(GV)) (Accession numbers: M35717(gb), 325405(gi)); HSVd.g2 (strain: German cultivar Riesling) (Accession numbers: X06873(embl), 60422(gi)); HSVd.g3 (strain: isolated from Vitis vinifera Rootstock 5BB) (Accession numbers: X15330(embl), 60648(gi)); HSVd.g4 (isolate grapevine (HSVdg), variant Ia) (Accession numbers: X87924(embl), 897764(gi)); HSVd.g5 (isolate grapevine (HSVdg), variant Ib) (Accession numbers: X87923(embl), 897765(gi)); HSVd.g6 (isolate grapevine (HSVdg), variant Ic) (Accession numbers: X87925(embl), 897766(gi)); HSVd.g7 (isolate grapevine (HSVdg), variant Id)(Accession numbers: X87926(embl), 897767(gi)); HSVd.g8 (isolate grapevine (HSVdg), variant Ie) (Accession numbers: X87927(embl), 897768(gi)); HSVd.g9 (isolate grapevine (HSVdg), variant IIa) (Accession numbers:X87928(embl), 897769(gi)); HSVd.cit1 (variant 1, isolate HSV-cit) (Accession numbers: X06718(embl), 60646(gi)); HSVd.cit2 (variant 2, isolate HSV-cit) (Accession numbers: X06719(embl), 60647(gi)); HSVd.cit3 (HSV.citrus) (Accession numbers: X13838(embl), 60418(gi)); HSVd.cit4(Accession numbers: U02527(gb), 409021(gi)); HSVd.cit5 (Hsu et al. *Virus Genes* **9**, 193-195 (1995)); HSVd.cit6 cit5 (Hsu et al. *Virus Genes* **9**, 193-195 (1995)); HSVd.cit7 (isolate CVd-IIa or E819) (Accession numbers: AF131248(gb)); HSVd.cit8 (isolate CVd-IIb or Ca902) (Accession numbers: AF131249(gb)); HSVd.cit9 (isolate CVd-IIc or Ca905) (Accession numbers: AF131250(gb)); HSVd.cit10 (isolate Ca903) (Accession numbers: AF131251(gb)); HSVd.cit11 (isolate CA909) (Accession

numbers: AF131252(gb)); HSVd.cit12 (cachexia isolate X-701-M) (Accession numbers: AF213483(gb), 12082502(gi)); HSVd.cit13 (cachexia isolate X-701-1) (Accession numbers: AF213484(gb), 12082503(gi)); HSVd.cit14 (cachexia isolate X-701-2) (Accession numbers: AF213485(gb), 12082504(gi)); HSVd.cit15 (cachexia isolate X-701-3) (Accession numbers: AF213486(gb), 12082505(gi)); HSVd.cit16 (cachexia isolate X-704-M) (Accession numbers: AF213487(gb), 12082506(gi)); HSVd.cit17 (cachexia isolate X-704-1) (Accession numbers: AF213488(gb), 12082507(gi)); HSVd.cit18 (cachexia isolate X-704-2) (Accession numbers: AF213489(gb), 12082508(gi)); HSVd.cit19 (cachexia isolate X-704-3) (Accession numbers: AF213490(gb), 12082509(gi)); HSVd.cit20 (cachexia isolate X-707-M) (Accession numbers: AF213491(gb), 12082510(gi)); HSVd.cit21 (cachexia isolate X-707-1) (Accession numbers: AF213492(gb), 12082511(gi)); HSVd.cit22 (cachexia isolate X-707-2) (Accession numbers: AF213493(gb), 12082512(gi)); HSVd.cit23 (cachexia isolate X-707-3) (Accession numbers: AF213494(gb), 12082513(gi)); HSVd.cit24 (cachexia isolate X-707-4) (Accession numbers: AF213495(gb), 12082514(gi)); HSVd.cit25 (cachexia isolate X-712-M) (Accession numbers: AF213496(gb), 12082515(gi)); HSVd.cit26 (cachexia isolate X-712-1) (Accession numbers: AF213497(gb), 12082516(gi)); HSVd.cit27 (cachexia isolate X-712-2) (Accession numbers: AF213498(gb), 12082517(gi)); HSVd.cit28 (cachexia isolate X-712-3) (Accession numbers: AF213499(gb), 12082518(gi)); HSVd.cit29 (cachexia isolate X-715-M) (Accession numbers: AF213500(gb), 12082519(gi)); HSVd.cit30 (cachexia isolate X-715-1) (Accession numbers: AF213501(gb), 12082520(gi)); HSVd.cit31 (cachexia isolate X-715-2) (Accession numbers: AF213502(gb), 12082521(gi)); HSVd.cit32 (CVd-Iia (117)) (Accession numbers: AF213503(gb), 12082522(gi)); HSVd.cit33 (isolate CVd-IIa 17uy) (Accession numbers: AF359276(gb), 13991644(gi)); HSVd.cit34 (isolate CVd-IIa 11uy) (Accession numbers: AF359275(gb), 13991643(gi)); HSVd.cit35 (isolate CVd-IIa 10uy) (Accession numbers: AF359274(gb), 13991642(gi)); HSVd.cit36 (isolate CVd-Ib 10uy) (Accession numbers:

AF359273(gb), 13991641(gi)); HSVd.cit37 (isolate CVd-Ib 5uy) (Accession numbers: AF359272(gb), 13991640(gi)); HSVd.cit38 (isolate CVd-Ib 3uy) (Accession numbers: AF359271(gb), 13991639(gi)); HSVd.cit39 (isolate CVd-Ib 2uy) (Accession numbers: AF359270(gb), 13991638(gi)); HSVd.cit40 (isolate CVd-IIa) (Accession numbers: X69519(embl), 2369773(gi)); HSVd.cit41 (isolate CVd-IIb) (Accession numbers: X69518(embl),2369774(gi)); HSVd.cit42 (isolate CVd-IIa 54-2-1) (Accession numbers: AF416554, 15811645(gi)); HSVd.cit43 (isolate CVd-IIa 54-2-2) (Accession numbers: AF416555, 15811646(gi)); HSVd.cit44 (isolate CVd-IIa 205-2-4) (Accession numbers: AF416556, 15811647(gi)); HSVd.cit45 (isolate CVd-IIa 205-2-1) (Accession numbers: AF416557, 15811648(gi)); HSVd.p1 (HSV-peach (A9)) (Accession numbers: D13765(dbj), 221254(gi)); HSVd.p2 (HSV-plum and HSV-peach (AF) isolate) (Accession numbers: D13764(dbj), 221255(gi)); HSVd.p3 (cv. Jeronimo J-16 from Spain) (Accession numbers: Y09352(embl),1684696(gi)); HSVd.apr1 (cv. Rouge de Roussillon from France) (Accession numbers: Y08438(embl), 2462494(gi)); HSVd.apr2 (unknown cultivar from Spain) (Accession numbers: Y08437 (embl), 2462495(gi)); HSVd.apr3 (cv. Bulida from Spain) (Accession numbers: Y09345(embl),1684690(gi)); HSVd.apr4 (cv. Bulida from Spain) (Accession numbers: Y09346(embl),1684691(gi)); HSVd.apr5 (cv. Bulida d'Arques from Spain) (Accession numbers: Y09344(embl),1684692(gi)); HSVd.apr6 (cv. Pepito del Rubio from Spain) (Accession numbers:Y09347(embl), 1684697(gi)); HSVd.apr7 (cv. Pepito del Rubio from Spain) (Accession numbers: 09348(embl), 1684699(gi)); HSVd.apr8 (cv. Pepito del Rubio from Spain) (Accession numbers: Y09349(embl), 684698(gi)); HSVd.apr9 (cv. Canino from Morocco) (Accession numbers: AJ297825(gb), 10944963(gi)); HSVd.apr10 (cv. Canino from Morocco) (Accession numbers: AJ297826(gb), 10944964(gi)); HSVd.apr11 (cv. Canino from Morocco) (Accession numbers: AJ297827(gb), 10944965(gi)); HSVd.apr12 (cv. Canino from Morocco) (Accession numbers: AJ297828(gb), 10944966(gi)); HSVd.apr13 (cv. Canino from Morocco) (Accession numbers:

AJ297829(gb), 10944967(gi)); HSVd.apr14 (cv. Septik from Turkey) (Accession numbers: AJ297830(gb), 10944968(gi)); HSVd.apr15 (cv. Monaco bello from Cyprus) (Accession numbers: AJ297831(gb), 10944969(gi)); HSVd.apr16 (cv.Cafona from Cyprus) (Accession numbers: AJ297832(gb), 10944970(gi)); HSVd.apr17 (cv.Cafona from Cyprus) (Accession numbers: AJ297833(gb), 10944971(gi)); HSVd.apr18 (cv.Boccuccia spinosa from Cyprus) (Accession numbers: AJ297834(gb), 10944972(gi)); HSVd.apr19 (cv. Palumella from Cyprus) (Accession numbers: AJ297835(gb), 10944973(gi)); HSVd.apr20 (cv. Palumella from Cyprus) (ccession numbers: AJ297836(gb), 10944974(gi)); HSVd.apr21 (cv.Canino from Cyprus) (Accession numbers: AJ297837(gb), 10944975(gi)); HSVd.apr22 (cv.Kolioponlou from Greece) (Accession numbers: AJ297838(gb), 10944976(gi)); HSVd.apr23 (cv. Bebecou Paros from Greece) (Accession numbers: AJ297839(gb), 10944977(gi)); HSVd.apr24 (cv. Bebecou Paros from Greece) (Accession numbers: AJ297840(gb), 10944978(gi)); HSVd.c1 (Cucumber pale fruit viroid (CPFVd), isolate HSV-cucumber) (Accession numbers: X00524(embl), 60644(gi)); HSVd.c2 (Cucumber pale fruit viroid (CPFVd)) (Accession numbers: X07405(embl), 59015(gi)); HSVd.alm1 (Accession numbers: AJ011813(emb), 3738118(gi)); HSVd.alm2 (Accession numbers: AJ011814(emb), 3738119(gi)); HSVd. Citrus viroid II, complete genome (Accession number: AF434679)]. All these nucleotide sequences are herein incorporated by reference.

As will be immediately apparent from the above list, viroids are extremely prone to sequence variations, and such natural variants can also be used for the currently described methods and means, particularly if they retain the capacity to be transported to the nucleus, together with any operably linked RNA.

In addition to the natural variations in viroid nucleotide sequences, variants may be obtained by substitution, deletion or addition of particular nucleotides, and such variants may also be suitable for the currently described methods and

means, particularly if they retain the capacity to be transported to the nucleus, together with any operably linked RNA.

Further, smaller RNA regions derived from the viroid nucleotide sequences, and variants thereof can be used for the current invention which are capable of being transported to the nucleus together with any operably linked RNA.

The capacity of both smaller regions and variants derived from viroid nucleotide sequences to be transported to the nucleus of a host cell, such as a plant cell, can be determined using the assay described by Zhou et al. 2001, J. Gen Virology, 82, 1491-1497. Briefly, the assay comprises introducing a marker coding region, such as GFP, comprising an intervening sequence in the coding region of the marker gene, into the host cell by means of a viral RNA vector that replicates in the cytoplasm of the host cell. When a functional nuclear localization signal is introduced (conveniently inserted in the intervening sequence), the viral RNA vector comprising the marker gene is imported into the nucleus, where the intron can be removed and the spliced RNA returned to the cytoplasm. The spliced RNA can be detected by the translation into GFP protein, as well as by RNA analysis methods (e.g. RT-PCR) to confirm the absence of the intron from the spliced RNA molecules.

Furthermore, the human hepatitis delta RNA is a 1700 nt single stranded circular RNA which is very similar to the viroids of the PSTVd-type in that is localized in the nucleus, forming rod-like structures, and may also be used according to the invention.

In another embodiment of the invention, the largely double stranded RNA region comprises CUG, CAG, GAC OR GUC repeats. As used herein « trinucleotide repeats or CUG, CAG, GAC OR GUC repeats » are RNA molecules comprising a number of CUG, CAG, GAC OR GUC trinucleotides. Preferably, the CUG trinucleotides are repeated without intervening sequences,

although short regions of 1 to 20-30 nucleotides not consisting of CUG trinucleotides may be present occasionally between the CUG trinucleotide repeats. Preferably, the CUG repeats comprise a number of CUG trinucleotide exceeding 35 copies or 44 copies such as any number between 50 and 2000 copies. Conveniently the copy number of the CUG triplets should not exceed 100 or 150. It is expected that CAG, GAC or GUC repeats may be used to similar effect.

Without intending to limit the invention to a particular mode of action, it is taught that such trinucleotide repeats repeats form rod-like structures by imperfect base-pairing which function as nuclear retention signal, possibly by sterically blocking RNA export through nuclear pores, as well as activate double stranded RNA dependent protein kinase PKR [Davis et al , 1997 Proc. Natl. Acad. Sci. 94, 7388-7393 ; Tian et al. 2000 RNA 6, 79-87 ; Koch and Lefert 1998 J. Theor. Biol. 192, 505-514).

CUG repeats may be particularly suited to increase the efficiency of antisense-mediated gene silencing when the RNA molecules comprising such CUG repeats can be delivered to the nucleus of the host cell e.g. through transcription of a chimeric gene encoding such RNA, as hereinafter described.

Although the largely double stranded RNA region such as the PSTVd-type viroid derived nuclear location signals or the trinucleotide repeats can conveniently be located at the 3' end of the target specific antisense RNA, it is expected that the location of the largely double stranded RNA is of little importance. Hence, largely double stranded RNA regions may also be located at the 5' end of the RNA molecule preferably at the 3' end or even in the middle of such an RNA molecule.

It was also unexpectedly found that the efficiency of antisense-mediated downregulation of gene expression, wherein the antisense RNA was operably

linked to a largely double stranded RNA region, could be further enhanced by inclusion of an intron sequence in the RNA molecule provided to the host cell. Again, the location of the intron in the RNA molecule with respect to both the target specific nucleotide sequence as well as the the largely double-stranded RNA region is expected to have little effect on the efficiency. In fact, it is expected that the largely double stranded RNA region may be located within the intron sequence.

As used herein, an "intron" or intervening sequence is used to refer to a DNA region within a larger transcribed DNA region, which is transcribed in the nucleus to yield an RNA region which is part of a larger RNA, however, said RNA region corresponding to intro sequence is removed from the larger RNA when transferred to the cytoplasm. The corresponding RNA is also referred to as an intron or intervening sequence. Intron sequences are flanked by splicing sites, and synthetic introns may be made by joining appropriate splice sites to basically any sequence, having an approriate branching point. Introns or intervening sequences which are located in 5'UTR, coding region or 3'UTR may be used.

Intervening sequences or introns should preferably be capable of being spliced in the eukaryotic host cells, although the presence of intervening sequences which can no longer be spliced, e.g. because their guide sequences have been altered or mutated, may even further increase the efficiency of the chimeric RNA molecules to down regulate the expression of a target gene. In one embodiment of the invention, the intron is essentially identical in sequence to the *Flaveria trinervia* pyruvate orthophosphate dikinase 2 intron 2 (pdk2 intron) and may comprise the sequence of SEQ ID No 9. Other examples of plant introns include the catalase intron from Castor bean (Accession number AF274974), the Delta12 desaturase (Fad2) intron from cotton (Accession number AF331163), the Delta 12 desaturase (Fad2) intron from *Arabidopsis* (Accession number AC069473), the Ubiquitin intron from maize (Accession

number S94464), the actin intron from rice. Other examples of mamalian virus introns include the intron from SV40. Examples of fungal introns include the intron from the triose phosphate isomerase gene from Aspergillus.

It was also unexpectedly found that further introduction of a sense RNA molecule with a target-gene specific region corresponding to the target gene specific region of the antisense RNA molecule already present in the cell of the eukaryotic organism, further increased the efficiency of the downregulation of the expression of the target gene. The same efficiency of downregulation of the expression of a target gene could be observed if the sense RNA molecule was provided with a largely double stranded RNA region as herein described. Sense RNA molecule was provided to a cell of a eukaryotic host organism simultaneously with an antisense RNA molecule capable of forming a double stranded region by basepairing with the sense RNA molecule.

Thus, in another embodiment of the invention a method is provided for down regulating the expression of a target gene in cells of a eukaryotic organisms, comprising the steps of

a) providing the cells of the eukaryotic organism with a first and second chimeric RNA molecule, wherein
   i) the first chimeric RNA molecule comprises an antisense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ;
   ii) the second chimeric RNA molecule comprises a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the complement of the first chimeric RNA molecule ;
   iii) the first and second chimeric RNA are capable of basepairing at least between the 19 consecutive nucleotides of the first chimeric

RNA and the 19 consecutive nucleotides of the second chimeric RNA ; and

iv) wherein either the first or the second chimeric RNA molecule comprises a largely double stranded RNA region operably linked to the antisense target-specific RNA region or to the sense target-specific RNA region ; and

b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

In another specific embodiment, both the first and second chimeric RNA molecule comprise a largely double stranded region. Specific embodiments for the largely double stranded RNA region and target gene-specific antisense RNA are as described elsewhere in this application. Specific embodiments for the sense RNA region are similar to the specific embodiments for the antisense RNA region.

Conveniently, the antisense or sense RNA molecules comprising a largely double stranded RNA region as herein described may be provided to the eukaryotic host cell or organism by introduction and possible integration of a chimeric gene, transcription of which yields such an antisense or sense RNA. Thus the invention is also aimed at providing such a chimeric gene comprising

- a promoter or a promoter region which is capable of being expressed in cells of the eukaryotic organism of interest; operably linked to a DNA region which when transcribed yields an antisense RNA molecule comprising
    - a target-gene specific antisense nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ; or
    - a target-gene specific sense nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene

identity with 19 consecutive nucleotides from the nucleotide sequence of the target gene ; operably linked to

- a largely double stranded RNA region as herein described ; and optionally
- a transcription termination and polyadenylation region suitable for the eukaryotic cell of choice.

As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (e.g., enhancers), at which gene expression regulatory proteins may bind.

The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (i.e., regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a DNA sequence located upstream (i.e., 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (i.e., 3') of the coding sequence and which comprises suitable transcription termination (and/or regulation) signals, including one or more polyadenylation signals.

In one embodiment of the invention the promoter is a constitutive promoter. In another embodiment of the invention, the promoter activity is enhanced by external or internal stimuli (inducible promoter), such as but not limited to hormones, chemical compounds, mechanical impulses, abiotic or biotic stress conditions. The activity of the promoter may also regulated in a temporal or spatial manner (tissue-specific promoters; developmentally regulated promoters).

In a particular embodiment of the invention, the promoter is a plant-expressible promoter. As used herein, the term "plant-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S (Hapster et al., 1988), the subterranean clover virus promoter No 4 or No 7 (WO9606932), or T-DNA gene promoters but also tissue-specific or organ-specific promoters including but not limited to seed-specific promoters (e.g., WO89/03887), organ-primordia specific promoters (An et al., 1996), stem-specific promoters (Keller et al., 1988), leaf specific promoters (Hudspeth et al., 1989), mesophyl-specific promoters (such as the light-inducible Rubisco promoters), root-specific promoters (Keller et al.,1989), tuber-specific promoters (Keil et al., 1989), vascular tissue specific promoters (Peleman et al., 1989), stamen-selective promoters (WO 89/10396, WO 92/13956), dehiscence zone specific promoters (WO 97/13865) and the like.

In another particular embodiment of the invention, the promoter is a fungus-expressible promoter. As used herein, the term "fungus-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in a fungal cell such as but not limited to the *A. nidulans trpC* gene promoter, or the inducible *S. cerevisiae* GAL4 promoter.

In yet another particular embodiment of the invention, the promoter is a animal-expressible promoter. As used herein, the term "animal-expressible promoter" means a DNA sequence which is capable of controlling (initiating) transcription in an animal cell and including but not limited to SV40 late and early promoters, cytomegalovirus CMV-IE promoters, RSV-LTR promoter, SCSV promoter, SCBV promoter and the like.

The antisense or sense RNA molecules useful for the invention may also be produced by *in vitro* transcription. To this end, the promoter of the chimeric genes according to the invention may be a promoter recognized by a bacteriophage single subunit RNA polymerase, such as the promoters recognized by bacteriophage single subunit RNA polymerase such as the RNA polymerases derived from the E. coli phages T7, T3, φI, φII, W31, H, Y, A1, 122, cro, C21, C22, and C2; Pseudomonas putida phage gh-1; Salmonella typhimurium phage SP6; Serratia marcescens phage IV; Citrobacter phage ViII; and Klebsiella phage No.11 [Hausmann, Current Topics in Microbiology and Immunology, 75: 77-109 (1976); Korsten et al., J. Gen Virol. 43: 57-73 (1975); Dunn et al., Nature New Biology, 230: 94-96 (1971); Towle et al., J. Biol. Chem. 250: 1723-1733 (1975); Butler and Chamberlin, J. Biol. Chem., 257: 5772-5778 (1982)]. Examples of such promoters are a T3 RNA polymerase specific promoter and a T7 RNA polymerase specific promoter, respectively. A T3 promoter to be used as a first promoter in the CIG can be any promoter of the T3 genes as described by McGraw et al, Nucl. Acid Res. 13: 6753-6766 (1985). Alternatively, a T3 promoter may be a T7 promoter which is modified at nucleotide positions -10, -11 and -12 in order to be recognized by T3 RNA polymerase [(Klement et al., J. Mol. Biol. 215, 21-29(1990)]. A preferred T3 promoter is the promoter having the "consensus" sequence for a T3 promoter, as described in US Patent 5,037,745. A T7 promoter which may be used according to the invention, in combination with T7 RNA polymerase, comprises a promoter of one of the T7 genes as described by Dunn and Studier, J. Mol. Biol. 166: 477-535 (1983). A preferred T7 promoter is the promoter having the "consensus" sequence for a T7 promoter, as described by Dunn and Studier (supra).

The antisense or sense RNA can be produced in large amounts by contacting the acceptor vector DNA with the appropriate bacteriophage single subunit RNA polymerase under conditions well known to the skilled artisan. The so-produced antisense or sense RNA can then be used for delivery into cells

prone to gene silencing, such as plant cells, fungal cells or animal cells. Antisense RNA may be introduced in animal cells via liposomes or other transfection agents (e.g. Clonfection transfection reagent or the CalPhos Mammalian transfection kit from ClonTech) and could be used for methods of treatment of animals, including humans, by silencing the appropriate target genes. Antisense or sense RNA can be introduced into the cell in a number of different ways. For example, the antisense or sense RNA may be administered by microinjection, bombardment by particles covered by the antisense or sense RNA, soaking the cell or organisms in a solution of the antisense or sense RNA, electroporation of cell membranes in the presence of antisense or sense RNA, liposome mediated delivery of antisense or sense RNA and transfection mediated by chemicals such as calcium phosphate, viral infection, transformation and the like. The antisense or sense RNA may be introduced along with components that enhance RNA uptake by the cell, stabilize the annealed strands, or otherwise increase inhibition of the target gene. In the case of a whole animal, the antisense or sense RNA is conveniently introduced by injection or perfusion into a cavity or interstitial space of an organism, or systemically via oral, topical, parenteral (including subcutaneous, intramuscular or intravenous administration), vaginal, rectal, intranasal, ophthalmic, or intraperitoneal administration. The antisense or sense RNA may also be administered via an implantable extended release device.

The chimeric genes according to the invention capable of producing antisense or sense RNA may also be equipped with any prokaryotic promoter suitable for expression of the antisense or sense RNA in a particular prokaryotic host. The prokaryotic host can be used as a source of antisense and/or sense RNA, e.g. by feeding it to an animal, such as a nematode or an insect, in which the silencing of the target gene is envisioned and monitored by reduction of the expression of the reporter gene. In this case, it will be clear that the target gene and reporter genes should be genes present in the cells of the target eukaryotic organism and not of the prokaryotic host organism. The antisense and sense

RNA according to the invention or chimeric genes capable of yielding such antisense or sense RNA molecules, can thus be produced in one host organism, be administered to a another target organisms (e.g. through feeding, orally administring, as a naked DNA or RNA molecule or encapsulated in a liposome, in a virus particle or attentuated virus particle, or on an inert particle etc.) and effect reduction of gene expression in the target gene or genes in another organism.

Suitable transcription termination and polyadenylation region include but are not limited to the SV40 polyadenylation signal, the HSV TK polyadenylation signal, the nopaline synthase gene terminator of *Agrobacterium tumefaciens*, the terminator of the CaMV 35S transcript, terminators of the subterranean stunt clover virus, the terminator of the *Aspergillus nidulans* trpC gene and the like.

The invention also aims at providing the antisense and sense RNA molecules, which may be obtained by transcription from these chimeric genes, and which are useful for the methods according to the invention.

It is another object of the invention to provide eukaryotic cells, and eukaryotic non-human organisms containing the antisense RNA molecules of the invention, or containing the chimeric genes capable of producing the antisense RNA molecules of the invention. In a preferred embodiment the chimeric genes are stably integrated in the genome of the cells of the eukaryotic organism.

It is also an object of the invention to provide eukaryotic cells and eukaryotic non-human organisms containing simultaneously sense and antisense RNA molecules of which one or both of the RNA molecules comprise a largely double stranded RNA region, or chimeric genes encoding such RNA molecules.

In another embodiment, the chimeric genes of the invention may be provided on a DNA molecule capable of autonomously replicating in the cells of the eukaryotic organism, such as e.g. viral vectors. The chimeric gene or the antisense or sense RNA may be also be provided transiently to the cells of the eukaryotic organism.

Introduction of chimeric genes (or RNA molecules) into the host cell can be accomplished by a variety of methods including calcium phosphate transfection, DEAE-dextran mediated transfection, electroporation, microprojectile bombardment, microinjection into nuclei and the like.

Methods for the introduction of chimeric genes into plants are well known in the art and include *Agrobacterium*-mediated transformation, particle gun delivery, microinjection, electroporation of intact cells, polyethyleneglycol-mediated protoplast transformation, electroporation of protoplasts, liposome-mediated transformation, silicon-whiskers mediated transformation etc. The transformed cells obtained in this way may then be regenerated into mature fertile plants.

Transgenic animals can be produced by the injection of the chimeric genes into the pronucleus of a fertilized oocyte, by transplantation of cells, preferably undifferentiated cells into a developing embryo to produce a chimeric embryo, transplantation of a nucleus from a recombinant cell into an enucleated embryo or activated oocyte and the like. Methods for the production of trangenic animals are well established in the art and include US patent 4, 873, 191 ; Rudolph et al. 1999 (Trends Biotechnology 17 :367-374) ; Dalrymple et al. (1998) Biotechnol. Genet. Eng. Rev. 15 : 33-49 ; Colman (1998) Bioch. Soc. Symp. 63 : 141-147 ; Wilmut et al. (1997) Nature 385 : 810-813, Wilmute et al. (1998) Reprod. Fertil. Dev. 10 : 639-643 ; Perry et al. (1993) Transgenic Res. 2 : 125-133 ; Hogan et al. Manipulating the Mouse Embryo, 2nd ed. Cold Spring Harbor Laboratory press, 1994 and references cited therein.

Gametes, seeds, embryos, progeny, hybrids of plants or animals comprising the chimeric genes of the present invention, which are produced by traditional breeding methods are also included within the scope of the present invention.

The methods and means described herein, can be applied to any eukaryotic organism in which gene-silencing takes place, including but not limited to plants (such as corn, wheat, potato, sunflower, turf grasses, barley, rye, tomato, sugar cane, safflower, cotton, *Arabidopsis*, rice, Brassica plants, vegetables, soybeans, tobacco, trees, flax, palm trees, peanuts, beans, etc.) invertebrate animals (such as insects, shellfish, molluscs, crustaceans such as crabs, lobsters and prawns) vertebrate animals (fish, avian animals, mammals, humans), yeast and fungi amongst others.

The following non-limiting Examples describe method and means for enhanced antisense RNA mediated silencing of the expression of a target gene in eukaryotic cell or combined sense/antisense RNA mediated target gene silencing.

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Other references for standard molecular biology techniques include Sambrook and Russell (2001) *Molecular Cloning: A Laboratory Manual*, Third Edition, Cold Spring Harbor Laboratory Press, NY, Volumes I and II of Brown (1998) *Molecular Biology LabFax*, Second Edition, Academic Press (UK). Standard materials and methods for polymerase chain reactions can be found in

Dieffenbach and Dveksler (1995) *PCR Primer: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) *PCR - Basics: From Background to Bench*, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences:

SEQ ID N°1: oligonucleotide primer for the amplication of the RG1 PSTVd

SEQ ID N°2: oligonucleotide primer for the amplication of the RG1 PSTVd

SEQ ID N°3: nucleotide sequence of the genome of PSTVd RG1

SEQ ID N°4: nucleotide sequence of genome of the Australian grapevine viroid

SEQ ID N°5: nucleotide sequence of the genome of the Coconut tinangaja viroid

SEQ ID N° 6: nucleotide sequence of the genome of the Tomato planta macho viroid

SEQ ID N°7: nucleotide sequence of the genome of the Hop latent viroid

SEQ ID N°8: nucleotide sequence of the genome of the Tomato apical stunt viroid

SEQ ID N°9: nucleotide sequence of the pdk2 intron

SEQ ID N°10: nucleotide sequence of the EIN2 cDNA

SEQ ID N° 11: nucleotide sequence the genomic EIN2 clone

SEQ ID N° 12: oligonucleotide primer 1 for the amplication of the EIN2 part used in the constructs in the Examples

SEQ ID N° 13: oligonucleotide primer 2 for the amplification of the EIN2 part used in the constructs in the Examples.

SEQ ID N° 14: pTSVd sequence in pMBW491.

SEQ ID N° 15: pTSVd sequence in pMBW489 (with 10 nt deletion).

Examples

Example 1 : Construction of the different plant lines containing different chimeric genes used.

As an example target gene to down-regulate the expression using the various constructs, the EIN2 gene from *Arabidopsis thaliana* was chosen. The down-regulation of the expression of the EIN2 gene can easily be visualized by germinating seeds on MS-ACC medium (containing aminocyclopropane-1-carboxylic acid (ACC)) and incubating either in the dark or in light.
Dark-grown EIN2 silenced seedlings grown in the dark have a longer hypocotyl and a more developed root system compared to wt seedlings, whereas EIN2 silenced seedlings grown in light can be differentiated from the wt seedlings by their larger cotyledon size (see Figure 3)

The EIN2 nucleotide sequence to be used in the different constructs in sense or antisense orientation was amplified by PCR using oligonucleotide primers with a nucleotide sequence as represented in SEQ ID N° 12 and 13 using genomic DNA (nucleotide sequence see SEQ ID N° 11) or cDNA (nucleotide sequence see SEQ ID N° 10) as template DNA. The amplication of the genomic EIN2 sequence part (gEIN2) resulted in a PCR fragment with the nucleotide sequence of SEQ ID N° 11 from the nucleotide at position 538 to the nucleotide at position 1123 and contains two native introns of the EIN2 gene.

The gEIN2 fragment was cloned as a KpnI/ClaI fragment into pART7 (Gleave, 1992 Plant. Mol. Biol. 20: 1203-1207), resulting in pMBW313 and the 35S promoter-gEIN2$_{sense}$-OCS3' cassette was cloned into pART27 (Gleave 1992 supra) at the NotI site to result in pMBW353.

A similar fragment (cEIN2) was amplified by PCR using EIN2 cDNA (SEQ ID N° 10) as template and the same pair of primers as for gEIN2. The cEIN2 fragment was digested with BamHI/ClaI and cloned into pSHUTTLE (Wang et

al., 1998 Acta Hort. 461 : 401-407) at the same sites, giving pMBW310. The cEIN2 fragment was then excised from pMBW310 with XbaI and cloned into the XbaI site of pART7, forming pMBW351. From this intermediate vector the 35S-EIN2antisense-OCS3' cassette was excised and cloned into pWBVec2A (Wang et al. 1998, supra) at the NotI site, resulting in pMBW360.

A full length sequence of the PSTVd strain RG1 (SEQ ID N° 3) was amplified from a cDNA using oligonucleotides with the nucleotide sequence of SEQ ID N°1 and SEQ ID N°2. The resulting PCR fragment was digested with BglII and cloned into the BamHI site of pMBW313, resulting in pMBW345, from which the 35S-gEIN2-PSTVd-OCS3' cassette was excised and cloned into pART27 at the NotI site resulting in pMBW355.

For pMBW359 the PCR amplified PSTVd sequence was digested with BglII and cloned into the BamHI site of pMBW310, giving pMBW346, from which the cEIN2antisense-PSTVd sequence was excised with XbaI and cloned into the XbaI site of pHANNIBAL (Wesley et al. 2001), forming pMBW349. The 35S-pdk2-cEIN2antisense-PSTVd-OCS3' cassette was then cloned into pWBVec2a at the NotI site forming pMBW359. The cEIN2antisense PSTVd fragment was also cloned into pWBVec2a to yield pMBW357.

The EIN2 cDNA fragment was excised form pMBW310 with EcoRV/BamHI, blunted by Pfu treatment and ligated into the BamHI site (also Pfu treated) of pKANNIBAL (Wesley et al. 2001). Plasmids having the cEIN2 in both orientations with respect to the 35S promoter were recovered and named pMWB401 (antisense) and pMBW404 (sense orientation).

For pLMW37, pLMW38, pLMW39, and pLMW40 the cEIN2 fragment was inserted in sense or antisense orientation upstream or downstream of an inverted repeat of the PSTVd sequence. To this end, a partial PSTVd sequence (SEQ ID N° 3 from the nucleotide at position 16 to the nucleotide at position

355) was cloned upstream of the pdk intron in inverse orientation with regard to the complete copy of the PSTVd genome.

The different constructs are schematically represented in Figure 2.

Example 2 : <u>Analysis of expression of the EIN2 gene in transgenic *Arabidopsis* lines comprising the different chimeric genes of Example 1.</u>

The chimeric constructs represented in Figure 2 were introduced into *Agrobacterium* tumefaciens using conventional methods and the resulting *Agrobacterium* strains were used to introduce the chimeric genes into *Arabidopsis* ecotype Landsberg erecta through the dipping method. Transgenic lines were selected on 15 mg/L hygromycin or 50 mg/L kanamycin as the selective agent. T1 opr F1 seed was collected and assayed for EIN2 silencing.

To this end, the seed was plated on MS medium containing 50μM ACC. The plates were sealed tightly with parafilm and kept either under light or in the dark. Silencing was scored by looking at the size of roots and cotyledons (incubation in the light) or by looking at the size of roots or hypocotyls (incubation in the dark). In EIN2 silenced lines, the roots or hypocotyls are significantly longer, and the cotyledons are significantly larger than in wt lines grown under the same conditions.

Seed from primary transformants was plated on MS-ACC medium, sealed with Parafilm, kept at 4C for 0-2 overnights, and then moved to growth room and kept either under light or in the dark. Silencing of the EIN2 gene was scored by examining the size of the roots and cotyledons (for those germinating under light) or the size of hypocotyls (for those in the dark). Significant or strongsilencing means long roots or hypocotyls, while weak silencing means bigger cotyledons but short roots or hypocotyls.The results are summarized in Table 1.

Table 1: Summary of the efficiency of EIN2 silencing in A. thaliana plants transformed with various EIN2 constructs.

Construct	Short description	transgenic lines	# strong silencing	# weak silencing	Frequency of silencing
PMBW360	• EIN2 antisense	23	2	5	30%
PMBW401	• EIN2 antisense • Pdk intron	20	0	3	15%
PMBW357	• EIN2 antisense • PSTVd	17	3	5	47%
PMBW359	• EIN2 antisense • PSTVd • Pdk intron	22	10	6	73%
PMBW353	• EIN2 sense • Native introns	19	2	3	26%
PMBW355	• EIN2 sense • Native introns • PSTVd	17	1	1	12%
PMBW404	• EIN 2 sense • PDK intron	20	3	2	25%
PLMW37	• EIN2 sense • Pdk intron • PSTVd repeat	19	0	0	0
PLMW38	• EIN2 antisense • Pdk intron • PSTVd repeat	10	1	2	30
PLMW39	• EIN2 sense • Pdk intron • PSTVd repeat	17	0	0	0
PLMW40	• EIN2 antisense • Pdk intron • PSTVd repeat	20	2	5	35%

Example 3: <u>Analysis of expression of the EIN2 gene in *Arabidopsis* lines obtained by crossing of the transgenic *Arabidopsis* lines comprising the different chimeric genes of Example 1.</u>

By cross-pollination between the *Arabidopsis* lines MBW353, MBW355, MBW359, MBW360 new lines were obtained containing simultaneously sense and antisense EIN2 constructs. These new lines were analyzed in a similar way

as described in Example 2. The results are summarized in Table 2. Plants wherein at least one of the transgenes contained a PSTVd sequence were very efficiently silenced.

Table 2. Summary of the efficiency of EIN2 silencing in A. thaliana plants comprising different combination of sense and antisense EIN2 constructs.

Line	Short description	N° of lines tested	N° of lines silenced	Frequency of silencing
MBW353 X MBW360	• EIN2 sense • Native introns And • EIN2 antisense	7	2	28.5%
MBW353 X MBW359	• EIN2 sense • Native introns And • EIN2 antisense • PSTVd • Pdk intron	3	3	100%
MBW355 X MBW360	• EIN2 sense • Native introns • PSTVd And • EIN2 antisense	5	4	80%
MBW355 X MBW359	• EIN2 sense • Native introns • PSTVd And • EIN2 antisense • PSTVd • Pdk intron	11	9	81.8%

Example 4 : Construction of different chimeric genes for mediating gene silencing of a GFP gene in mammalian cells and analysis in CHO cells.

As an example target gene to down-regulate the expression in mammalian cells, the humanized GFP coding region, expressed under control of a CMV promoter region, and followed by a SV40 polyadenylation signal was chosen (pCI-GFP)

Different experimetal silencing constructs were constructed, having either the GFP coding region cloned in sense (as in pMBW493, pMBW494 and pMBW497) or antisense orientation (as in pMBW489, pMBW491 or pMBW496) with regard to the CMV promoter region.

Plasmids pMBW493 and pMBW489 contained downstream of the GFP coding region, but upstream of the SV40 polyadenylation signal, a nucleotide seqeunce corresponding to a PSTVd sequence but with a 10 nt deletion (SEQ ID No 15). This deletion has an impact on the predicted secondary structure (see Fig 5).

Plasmids pMBW494 and pMBW491 contained downstream of the GFP coding region, but upstream of the SV40 polyadenylation signal, a nucleotide seqeunce corresponding to a PSTVd sequence of SEQ ID No 14 without the 10 nt deletion.

Plasmids pMBW497 and pMBW496 contained downstream of the GFP coding region, but upstream of the SV40 polyadenylation signal, a nucleotide sequence comprising 60 CUG trinucleotide repeats.

The different experimental plasmids were introduced (at different concentrations) into CHO cells in combination with a plasmid comprising the

GFP expressing chimeric gene (Table 3; entries 1 to 18). Since the GFP construct is a functional sequence in the sense constructs, sense GFP containing experimental constructs were also introduced without the extra GFP expressing chimeric gene; to estimate the GFP expression by these constructs alone (Table 3; entries 19 to 30). Further, combinations of antisense and sense experimental constructs were introduced in CHO cells, at different concentrations(Table 3; entries 31 to 42). As a control, the chimeric GFP expression construct (pCi-GFP) was introduced alone into CHO cells.

After 24 hrs or 48 hrs, the cells were assayed for GFP expression. Average counts and standard deviations are represented in Table 3.

The antisense GFP constructs pMBW491, pMBW496 and pMBW489 that carry the pTSVd or CUG repeat sequences resulted in a significant reduction of the expression of the GFP gene.

Interestingly, pMWB489 in which the PSTVd sequence contains a 10 nt deletion, resulted in slower and lower degrees of GFP silencing than pMWB491, which contains an intact PSTVd sequence.

	Experimental DNA	Target DNA	Remarks on Experimental DNA	Average count (24 hr)	Standard deviation	Average count (48 hr)	Standard deviation
1	0.1μg pMBW89	0.3μg GFP	Antisense+PSTVd (deletion)	3626	206	9058	1468
2	0.3μg pMBW 89	0.3μg GFP		3521	41	6468	3522
3	0.7μg pMBW 89	0.3μg GFP		3167	1348	1096	2191
4	0.1μg pMBW 91	0.3μg GFP	Antisense+PSTVd	3585	86	5908	623
5	0.3μg pMBW 91	0.3μg GFP		748	128	1426	332.3
6	0.7μg pMBW 91	0.3μg GFP		23	25	1637	70
7	0.1μg pMBW 96	0.3μg GFP	Antisense + CUG repeats	3217	467	5221	4700
8	0.3μg pMBW 96	0.3μg GFP		2907	107	3272	0
9	0.7μg pMBW 96	0.3μg GFP		181	92	1433	466
10	0.1μg pMBW 93	0.3μg GFP	Sense + PSTVd (deletion)	5815	313	16482	470
11	0.3μg pMBW 93	0.3μg GFP		10453	1555	15810	1067
12	0.7μg pMBW 93	0.3μg GFP		12718	5423	10666	949
13	0.1μg pMBW 94	0.3μg GFP	Sense+PSTVd	9166	1269	15023	263
14	0.3μg pMBW 94	0.3μg GFP		12719	3894	6699	94
15	0.7μg pMBW 94	0.3μg GFP		1009	658	13133	824
16	0.1μg pMBW 97	0.3μg GFP	Sense+CUG repeats	6414	1367	15795	178
17	0.3μg pMBW 97	0.3μg GFP		3596	50	10235	770
18	0.7μg pMBW 97	0.3μg GFP		729	295	13171	2868
19	0.1μg pMBW 93	None	Sense + PSTVd (deletion)	1216	15	3692	142
20	0.3μg pMBW 93	None		6022	1293	9341	273
21	0.5μg pMBW 93	None		6795	3235	11466	2541
22	0.7μg pMBW 93	None		12002	763	10316	1523
23	0.1μg pMBW 94	None	Sense+PSTVd	2121	594	5417	777
24	0.3μg pMBW 94	None		5671	5096	9317	743
25	0.5μg pMBW 94	None		6349	3253	7842	337
26	0.7μg pMBW 94	None		1785	729	15574	2208
27	0.1μg pMBW 97	None	Sense+CUG repeats	4448	626	6064	289
28	0.3μg pMBW 97	None		487	83	7767	194
29	0.5μg pMBW 97	None		522	223	7481	566
30	0.7μg pMBW 97	None		270	159	8980	1154

	Experimental DNA	Target DNA	Remarks on Experimental DNA	Average count (24 hr)	Standard deviation	Average count (48 hr)	Standard deviation
31	0.1μg pMBW 93 + 0.1μg pMBW 91	None	Sense + PSTVd (deletion) and	1189	148	2331	815
32	0.3μg pMBW 93 + 0.3μg pMBW 91	None	Antisense+PSTVd	695	83	3101	533
33	0.5μg pMBW 93 + 0.5μg pMBW 91	None		111	117	3758	1583
34	0.3μg pMBW 93 + 0.1μg pMBW 91	None		1811	1304	5301	73
35	0.3μg pMBW 93 + 0.3μg pMBW 91	None		312	171	4972	401
36	0.3μg pMBW 93 + 0.7μg pMBW 91	None		14	20	2896	1075
37	0.1μg pMBW 97 + 0.1μg pMBW 96	None	Antisense+CUG repeats and	3841	929	2945	341
38	0.3μg pMBW 97 + 0.3μg pMBW 96	None	Sense+CUG repeats	1018	401	3236	822
39	0.5μg pMBW 97 + 0.5μg pMBW 96	None		1262	241	6730	289
40	0.3μg pMBW 97 + 0.1μg pMBW 96	None		3603	2785	10349	3463
41	0.3μg pMBW 97 + 0.3μg pMBW 96	None		4903	1054	3453	2380
42	0.3μg pMBW 97 + 0.7μg pMBW 96	none		278	46	5897	1899
43	**None**	**0.3μg GFP**	**Control**	**4780**	**688**	**25175**	**8289.6**

Table 3. Summary of GFP expression in into CHO cells.transformed by the different experimental constructs.

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We claim

1) A method for down regulating the expression of a target gene in cells of a eukaryotic organisms, comprising the steps of
   a) providing the cells of the eukaryotic organism with a chimeric RNA molecule wherein said chimeric RNA molecule comprises
      i) a target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene; operably linked to
      ii) a largely double stranded RNA region ; and
   b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

2) The method according to claim 1, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type.

3) The method according to claim 2, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

4) The method according to claim 3, wherein said viroid has a genome nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

5) The method according to any one of claims 2 to 4, wherein said nuclear localization signal is from Potato spindle tuber viroid.

6) The method according to any one of claims 2 to 5, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

7) The method according to any one of claims 2 to 6, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

8) The method according to claim 2 or 3, wherein said largely double stranded RNA comprises a viroid genome nucleotide sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

9) The method according to claim 8, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

10)The method according to any one of claims 2 to 9, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

11)The method according to claim 10, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

12)The method of claim 11, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

13)The method according to claim 1, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

14)The method according to claim 13, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

15)The method according to any one of claims 1 to 14, wherein said RNA molecule comprises multiple target-gene specific regions.

16)The method according to any one of claims 1 to 15, wherein said RNA molecule comprises an intron sequence.

17)The method according to claim 16, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean, the Delta12 desaturase intron from cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

18) The method according to any one of claims 1 to 17 wherein said eukaryotic organism is a plant.

19) The method according to claim 18, wherein said plant is selected from the group of *Arabidopsis*, alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, wheat, asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucmber, eggplant, lettuce, onion, oilseed rape, pepper, potato, pumpkin, radish, spinach, squash, tomato, zucchini, almond, apple, apricot, banana, blackberry, b lueberry, cacao, cherry, coconut, cranberry, date, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut and watermelon.

20) The method according to any one of claims 1 to 17, wherein said eukaryotic organism is a fungus, yeast or mold.

21) The method according to any one of claims 1 to 17, wherein said eukaryotic organism is an animal.

22) The method according to claim 21, wherein said animal is a human, mammal, fish, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, guinea pig, rabbit, primate, nematode, shellfish, prawn, crab, lobster, insect, fruit fly, Coleapteran insect, Dipteran insect, Lepidopteran insect and Homeopteran insect.

23) The method according to any one of claims 1 to 22, wherein said chimeric RNA is produced by transcription from a chimeric DNA molecule.

24) A chimeric RNA molecule for down-regulating the expression of a target gene in a cell of a eukaryotic organisms, comprising

a) a target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of said target gene in said cell of said eukaryotic organism ; operably linked to

b) a largely double stranded RNA region ;

wherein said chimeric RNA molecule, when provided to cells of said eukaryotic organism down-regulates the expression of said target gene.

25)The chimeric RNA molecule according to claim 24, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)type.

26)The chimeric RNA molecule according to claim 25, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

27)The chimeric RNA molecule according to claim 25 or 26, wherein said viroid has a genome nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

28)The chimeric RNA molecule according to any one of claims 25 or 26, wherein said nuclear localization signal is from Potato spindle tuber viroid.

29)The chimeric RNA molecule according to any one of claims 25 or 26, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

30)The chimeric RNA molecule according to any one of claims 25 to 29, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

31)The chimeric RNA molecule according to claim 25 or 26, wherein said largely double stranded RNA comprises a viroid genome nucleotide sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

32)The chimeric RNA molecule according to claim 31, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

33)The chimeric RNA molecule according to any one of claims 25 to 32, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

34)The chimeric RNA molecule according to claim 33, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

35)The chimeric RNA molecule of claim 34, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

36)The chimeric RNA molecule according to claim 24, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

37)The chimeric RNA molecule according to claim 36, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

38)The chimeric RNA molecule according to any one of claims 24 to 37, wherein said RNA molecule comprises multiple target-gene specific regions.

39)The chimeric RNA molecule according to any one of claims 24 to 38, wherein said RNA molecule comprises an intron sequence.

40)The chimeric RNA molecule according to claim 39, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean, the Delta12 desaturase intron from cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

41) A chimeric DNA molecule for reduction of the expression of a target gene in a cell of a eukaryotic organism, comprising

a) a promoter or promoter region capable of being recognized by RNA polymerases in said cells of said eukaryotic organism ; operably linked to

b) a DNA region, which when transcribed yields an RNA molecule, said RNA molecule comprising

i) a target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of said target gene in said cell of said eukaryotic organism ; operably linked to

ii) a largely double stranded RNA region ;

wherein said chimeric DNA molecule, when provided to cells of said eukaryotic organism reduces the expression of said target gene.

42) The chimeric DNA molecule according to claim 41, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the potato spindle tuber viroid type.

43) The chimeric DNA molecule according to claim 42, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

44) The chimeric DNA molecule according to claim 42 or 43, wherein said viroid has a genome nucleotide sequence selected from the group

consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

45)The chimeric DNA molecule according to any one of claims 42 to 44, wherein said nuclear localization signal is from Potato spindle tuber viroid.

46)The chimeric DNA molecule according to claim 45, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

47)The chimeric DNA molecule according to any one of claims 42 to 46, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

48)The chimeric DNA molecule according to claim 42 or 43, wherein said largely double stranded RNA comprises a viroid genome nucleotide sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

49)The chimeric DNA molecule according to claim 48, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID

N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

50)The chimeric DNA molecule according to any one of claims 42 to 49, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

51)The chimeric DNA molecule according to claim 50, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

52)The chimeric DNA molecule of claim 51, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

53)The chimeric DNA molecule according to claim 41, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

54)The chimeric DNA molecule according to claim 53, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

55)The chimeric DNA molecule according to any one of claims 41 to 54, wherein said RNA molecule comprises multiple target-gene specific regions.

56)The chimeric DNA molecule according to any one of claims 41 to 55, wherein said RNA molecule comprises an intron sequence.

57)The chimeric DNA molecule according to claim 56, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean; the Delta12 desaturase intron from

cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

58)The chimeric DNA molecule according to any one of claims 41 to 56, further comprising a transcription termination and polyadenylation signal operably linked to said DNA region encoding said RNA molecule.

59)The chimeric DNA molecule according to any one of claims 41 to 58, wherein said promoter or promoter region is a plant-expressible promoter.

60)The chimeric DNA molecule according to any one of claims 41 to 58, wherein said promoter or promoter region is a promoter which functions in animals.

61)The chimeric DNA molecule according to any one of claims 41 to 58, wherein said promoter or promoter region is a promoter which functions in yeast, fungi or molds.

62)The chimeric DNA molecule according to any one of claims 41 to 58, wherein said promoter or promoter region is promoter recognized by a single subunit bacteriophage RNA polymerase.

63)A cell from a eukaryotic organism comprising a chimeric DNA molecule according to any one of claims 41 to 62.

64)A eukaryotic cell comprising a chimeric RNA molecule according to any one of claims 24 to 40.

65)The cell according to claim 63 or claim 64, wherein said eukaryotic organism is a plant.

66)The cell according to claim 65, wherein said plant is selected from the group of *Arabidopsis*, alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, wheat, asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucmber, eggplant, lettuce, onion, oilseed rape, pepper, potato, pumpkin, radish, spinach, squash, tomato, zucchini, almond, apple, apricot, banana, blackberry, b lueberry, cacao, cherry, coconut, cranberry, date, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut and watermelon.

67)The cell according to claim 63 or claim 64, wherein said eukaryotic organism is a fungus, yeast or mold.

68)The cell according to claim 63 or claim 64, wherein said eukaryotic organism is an animal.

69)The cell according to claim 68, wherein said animal is a human, mammal, fish, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, guinea pig, rabbit, primate, nematode, shellfish, prawn, crab, lobster, insect, fruit fly, Coleapteran insect, Dipteran insect, Lepidopteran insect and Homeopteran insect.

70)A non-human eukaryotic organism, comprising in its cells a chimeric DNA molecule according to any one of claims 41 to 62.

71)A non-human eukaryotic organism, comprising in its cells a chimeric RNA molecule according to any one of claims 24 to 40.

72) The non-human eukaryotic organism according to claim 70 or claim 71, wherein said eukaryotic organism is a plant.

73) The non-human eukaryotic organism according to claim 72, wherein said plant is selected from the group of *Arabidopsis*, alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, wheat, asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucmber, eggplant, lettuce, onion, oilseed rape, pepper, potato, pumpkin, radish, spinach, squash, tomato, zucchini, almond, apple, apricot, banana, blackberry, b lueberry, cacao, cherry, coconut, cranberry, date, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut and watermelon.

74) The non-human eukaryotic organism according to claim 70 or claim 71, wherein said eukaryotic organism is a fungus, yeast or mold.

75) The non-human eukaryotic organism according to claim 70 or claim 71, wherein said eukaryotic organism is an animal.

76) The non-human eukaryotic organism according to claim 75, wherein said animal is a human, mammal, fish, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, guinea pig, rabbit, primate, nematode, shellfish, prawn, crab, lobster, insect, fruit fly, Coleapteran insect, Dipteran insect, Lepidopteran insect and Homeopteran insect.

77) Use of a chimeric RNA molecule according to any one of claims 24 to 40 for reduction of the expression of a target gene in a cell of a eukaryotic organism.

78) Use of a chimeric DNA molecule according to any one of claims 41 to 62 for reduction of the expression of a target gene in a cell of a eukaryotic organism.

79) A method for making a transgenic eukaryotic organism wherein expression of a target gene in cells of said organism is reduced, said method comprising the steps of :

a) providing a chimeric DNA molecule according to any one of claims 41 to 62 to a cell or cells of said organism to make a transgenic cell or cells ;
b) growing or regenerating a transgenic eukaryotic organism from said transgenic cell or cells.

80) A method for down regulating the expression of a target gene in cells of a eukaryotic organisms, comprising the steps of

a) providing the cells of the eukaryotic organism with a first and second chimeric RNA molecule,
   i) said first chimeric RNA molecule comprising an antisense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ;
   ii) said second chimeric RNA molecule comprising a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the complement of said first chimeric RNA molecule ;
   iii) said first and second chimeric RNA being capable of basepairing at least between said 19 consecutive nucleotides of said first chimeric RNA and said 19 consecutive nucleotides of said second chimeric RNA ; and
   iv) wherein either said first or said second chimeric RNA molecule comprises a largely double stranded RNA region operably linked to

said antisense target-specific RNA region or to said sense target-specific RNA region ; and

b) identifying those eukaryotic organisms wherein the expression of the target gene is down regulated.

81) The method according to claim 80, wherein said first and said second chimeric RNA molecule comprise a largely double stranded RNA region.

82) The method according to claim 81, wherein said first and said second chimeric RNA molecule comprise the same largely double stranded RNA region.

83) The method according to any one of claims 80 to 82, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type.

84) The method according to claim 83, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

85) The method according to claim 83, wherein said viroid has a genome nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

86) The method according to any one of claims 83 to 85, wherein said nuclear localization signal is from Potato spindle tuber viroid.

87)The method according to any one of claims 83 to 86, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

88)The method according to any one of claims 83 to 87, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

89)The method according to claim 83 or 84, wherein said largely double stranded RNA comprises a viroid genome nucleotide sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

90)The method according to claim 89, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

91)The method according to any one of claims 83 to 90, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

92) The method according to claim 91, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

93) The method of claim 92, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

94) The method according to any one of claims 80 to 82, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

95) The method according to claim 94, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

96) The method according to any one of claims 80 to 95, wherein said RNA molecule comprises multiple target-gene specific regions.

97) The method according to any one of claims 80 to 96, wherein said RNA molecule comprises an intron sequence.

98) The method according to claim 97, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean, the Delta12 desaturase intron from cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

99) The method according to any one of claims 80 to 98, wherein said first and second chimeric RNA are transcribed from a first and second chimeric gene.

100) A cell from a eukaryotic organism comprising a first and second chimeric RNA molecule,

i) said first chimeric RNA molecule comprising an antisense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity with the complement of 19 consecutive nucleotides from the nucleotide sequence of the target gene ;

ii) said second chimeric RNA molecule comprising a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the complement of said first chimeric RNA molecule ;

iii) said first and second chimeric RNA being capable of basepairing at least between said 19 consecutive nucleotides of said first chimeric RNA and said 19 consecutive nucleotides of said second chimeric RNA ; and

iv) wherein either said first or said second chimeric RNA molecule comprises a largely double stranded RNA region operably linked to said antisense target-specific RNA region or to said sense target-specific RNA region .

101) The cell according to claim 100, wherein said first and said second chimeric RNA molecule comprise a largely double stranded RNA region.

102) The cell according to claim 101, wherein said first and said second chimeric RNA molecule comprise the same largely double stranded RNA region.

103) The cell according to any one of claims 100 to 102, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type.

104) The cell according to claim 103, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

105) The cell according to claim 103, wherein said viroid has a genome nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

106) The cell according to any one of claims 103 to 105, wherein said nuclear localization signal is from Potato spindle tuber viroid.

107) The cell according to any one of claims 103 to 106, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

108) The cell according to any one of claims 103 to 107, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

109) The cell according to claim 103 or 104, wherein said largely double stranded RNA comprises a viroid genome nucleotide sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence

of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

110) The cell according to claim 109, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

111) The cell according to any one of claims 103 to 109, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

112) The cell according to claim 111, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

113) The cell of claim 112, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

114) The cell according to any one of claims 100 to 102, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

115) The cell according to claim 114, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

116) The cell according to any one of claims 100 to 115, wherein said RNA molecule comprises multiple target-gene specific regions.

117) The cell according to any one of claims 100 to 116, wherein said RNA molecule comprises an intron sequence.

118) The cell according to claim 117, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean, the Delta12 desaturase intron from cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

119) The cell according to any one of claims 100 to 118 wherein said first and second chimeric RNA are transcribed from a first and second chimeric gene.

120) A non-human eukaryotic organism comprising the cell according to any one of claims 100 to claim 119 .

121) A chimeric sense RNA molecule for reduction of expression of a target gene in a cell of a eukaryotic organism in cooperation with a chimeric antisense RNA molecule, said chimeric sense RNA molecule comprising

a) a sense target-gene specific RNA region comprising a nucleotide sequence of at least about 19 consecutive nucleotides having at least about 94% sequene identity to the nucleotide of said target gene ; operably linked to

b) a largely double stranded RNA region.

122) The chimeric RNA molecule according to claim 121, wherein the largely double stranded RNA region comprises a nuclear localization signal from a viroid of the Potato spindle tuber viroid (PSTVd)-type.

123) The chimeric RNA molecule according to claim 122, wherein said nuclear localization signal is from a viroid selected from the group consisting of Potato Spindle tuber viroid, Citrus viroid species III, Citrus viroid species IV, Hop latent viroid, Australian grapevine viroid, Tomato planta macho viroid, Coconut tinangaja viroid, Tomato apical stunt viroid, Coconut cadang-cadang viroid, Citrus exocortis viroid, Columnea latent viroid, Hop stunt viroid and Citrus bent leaf viroid.

124) The chimeric RNA molecule according to claim 123, wherein said viroid has a genome nucleotide sequence selected from the group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

125) The chimeric RNA molecule according to any one of claims 122 to 124, wherein said nuclear localization signal is from Potato spindle tuber viroid.

126) The chimeric RNA molecule according to any one of claims 122 to 125, wherein said nuclear localization signal is from Potato spindle viroid strain RG1.

127) The chimeric RNA molecule according to any one of claims 122 to 126, wherein said nuclear localization signal comprises a nucleotide sequence functioning as a nuclear localization signal selected from the nucleotide sequence of SEQ ID N° 3.

128) The chimeric RNA molecule according to claim 122 or 123, wherein said largely double stranded RNA comprises a viroid genome nucleotide

sequence selected from the group consisting of the genome nucleotide sequence of Potato Spindle tuber viroid, the genome nucleotide sequence of Citrus viroid species III, the genome nucleotide sequence of Citrus viroid species IV, the genome nucleotide sequence of Hop latent viroid, the genome nucleotide sequence of Australian grapevine viroid, the genome nucleotide sequence of Tomato planta macho viroid, the genome nucleotide sequence of Coconut tinangaja viroid, the genome nucleotide sequence of Tomato apical stunt viroid, the genome nucleotide sequence of Coconut cadang-cadang viroid, the genome nucleotide sequence of Citrus exocortis viroid, the genome nucleotide sequence of Columnea latent viroid, the genome nucleotide sequence of Hop stunt viroid and the genome nucleotide sequence of Citrus bent leaf viroid.

129) The chimeric RNA molecule according to claim 128, wherein said viroid genome nucleotide sequence is selected from group consisting of SEQ ID N° 3, SEQ ID N° 4, SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7 and SEQ ID N° 8.

130) The chimeric RNA molecule according to any one of claims 122 to 129, wherein said largely double stranded RNA region comprises a genomic nucleotide sequence of Potato spindle tuber viroid.

131) The chimeric RNA molecule according to claim 130, wherein said viroid genome nucleotide sequence is the genome nucleotide sequence of Potato spindle tuber viroid strain RG1.

132) The chimeric RNA molecule of claim 131, wherein said genome nucleotide sequence has the nucleotide sequence of SEQ ID N° 3.

133) The chimeric RNA molecule according to claim 121, wherein said largely double stranded RNA region comprises at least 35 repeats of the trinucleotide CUG.

134) The chimeric RNA molecule according to claim 133, wherein said largely double stranded RNA region comprises between 44 and 2000 repeats of the trinucleotide CUG.

135) The chimeric RNA molecule according to any one of claims 121 to 134, wherein said RNA molecule comprises multiple target-gene specific regions.

136) The chimeric RNA molecule according to any one of claims 121 to 135, wherein said RNA molecule comprises an intron sequence.

137) The chimeric RNA molecule according to claim 136, wherein said intron sequence is selected from the group consisting of the pdk2 intron, the catalase intron from Castor bean, the Delta12 desaturase intron from cotton, the Delta 12 desaturase intron from *Arabidopsis*, the Ubiquitin intron from maize, the Actin intron from rice, the triose phosphate isomerase intron from Aspergillus and the intron from SV40.

138) A chimeric DNA molecule for reduction of the expression of a target gene in a cell of a eukaryotic organism, comprising

a) a promoter or promoter region capable of being recognized by RNA polymerases in said cells of said eukaryotic organism ; operably linked

b) a DNA region, which when transcribed yields a chimeric sense RNA molecule as described in any one of claim 121 to 137.

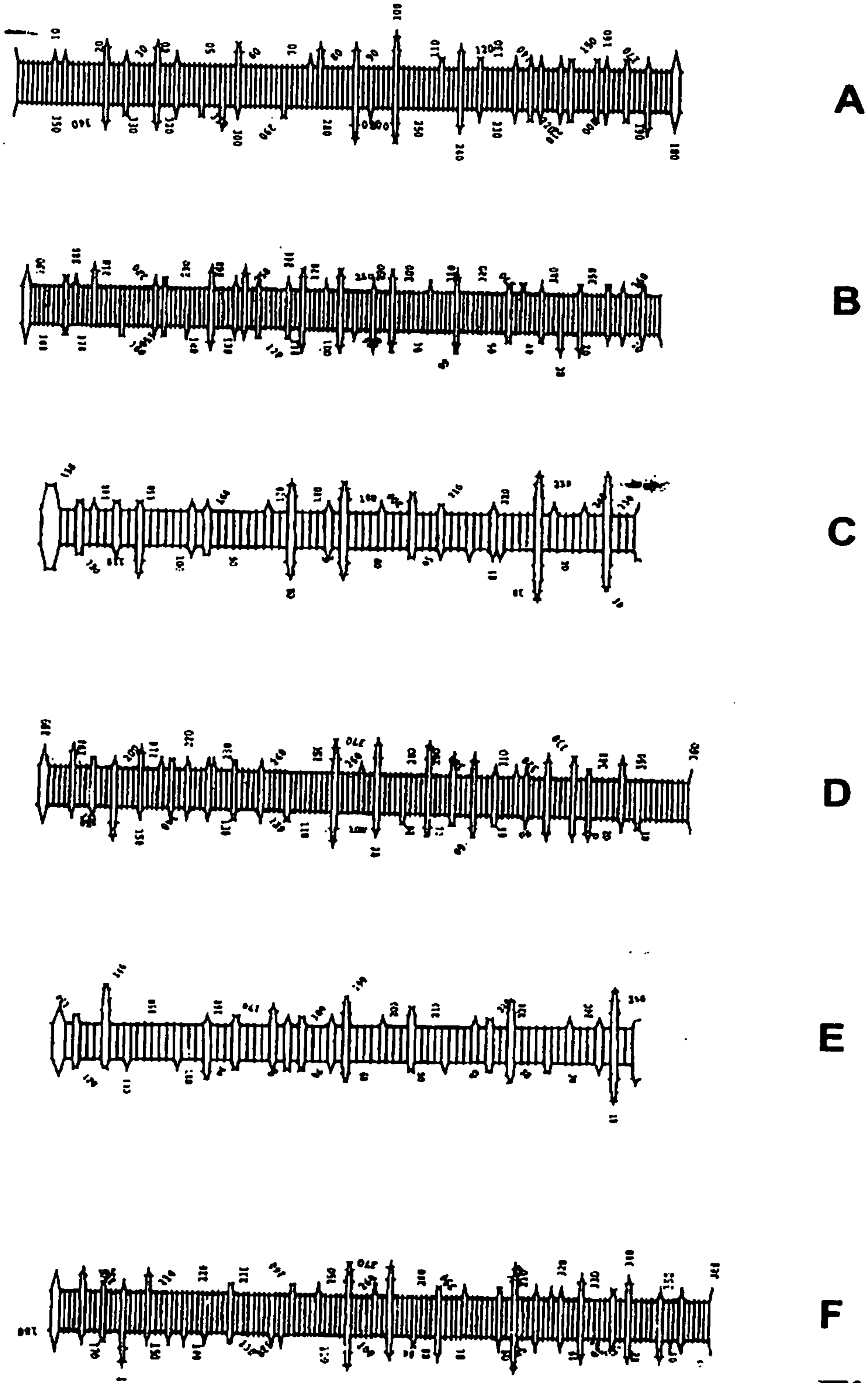
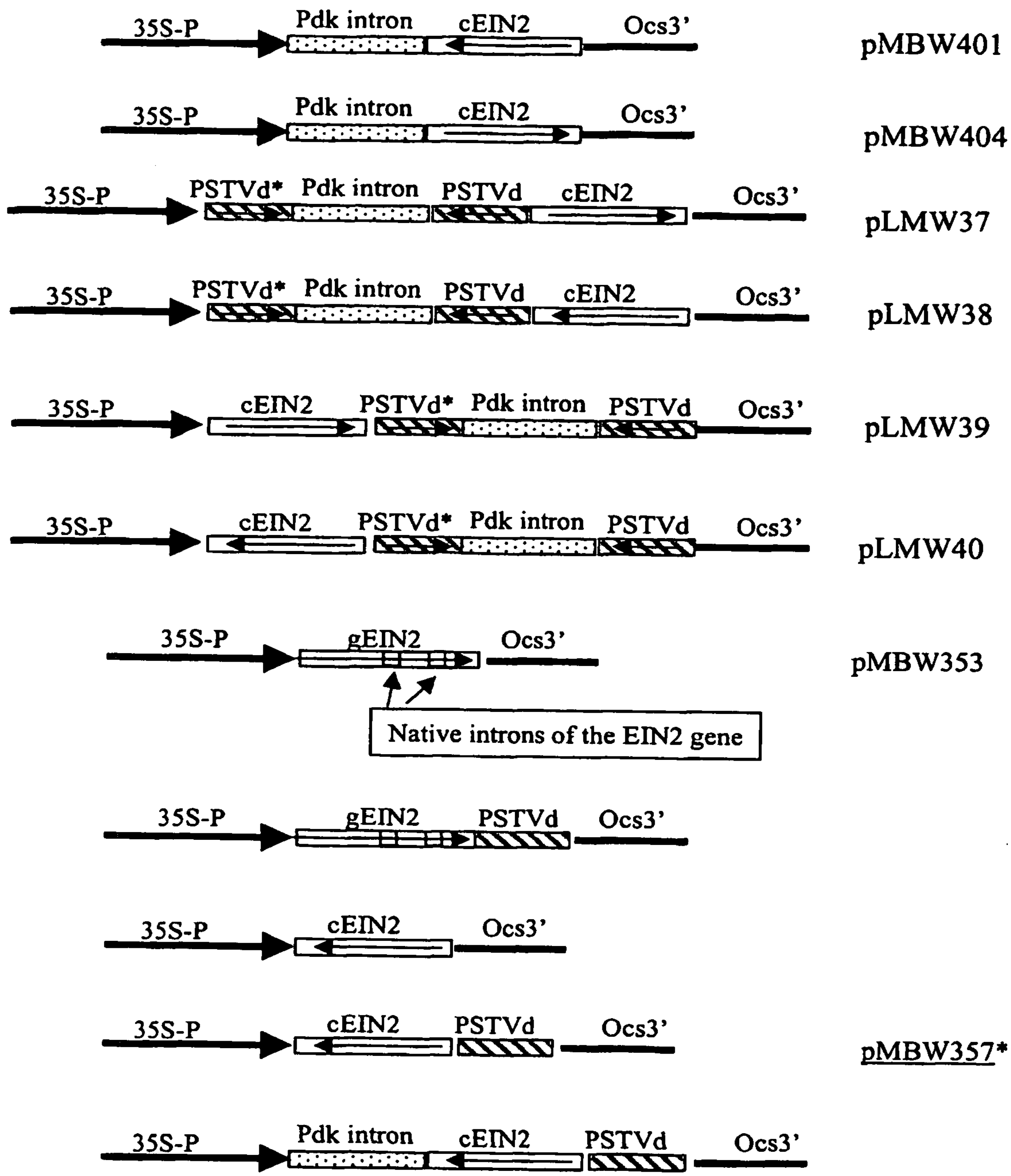


Figure 1


35S-P
Pdk intron
cEIN2
Ocs3'
pMBW401
35S-P
Pdk intron
cEIN2
Ocs3'
pMBW404
35S-P
PSTVd*
Pdk intron
PSTVd
cEIN2
Ocs3'
pLMW37
35S-P
PSTVd*
Pdk intron
PSTVd
cEIN2
Ocs3'
pLMW38
35S-P
cEIN2
PSTVd*
Pdk intron
PSTVd
Ocs3'
pLMW39
35S-P
cEIN2
PSTVd*
Pdk intron
PSTVd
Ocs3'
pLMW40
35S-P
gEIN2
Ocs3'
pMBW353
Native introns of the EIN2 gene
35S-P
gEIN2
PSTVd
Ocs3'
35S-P
cEIN2
Ocs3'
35S-P
cEIN2
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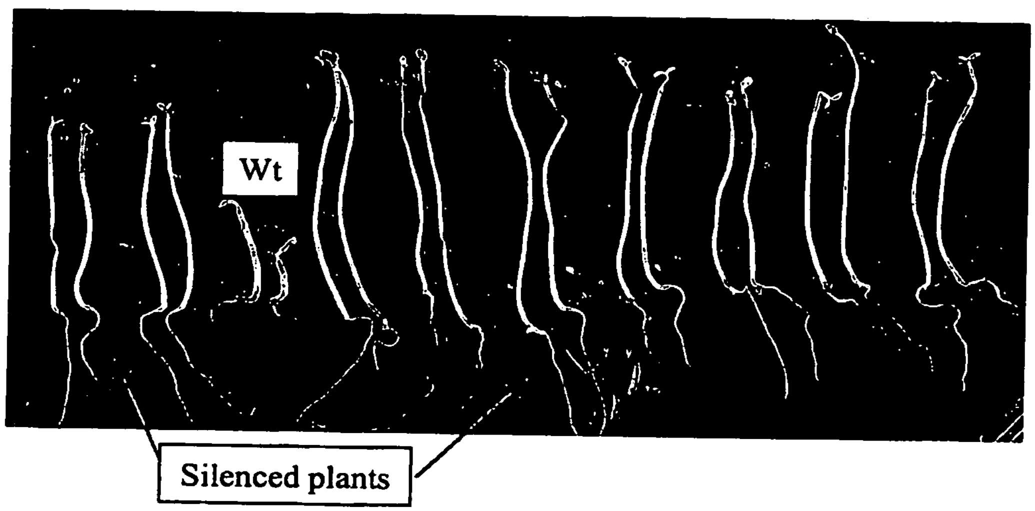
PSTVd
Ocs3'

Figure 2

A.



B.

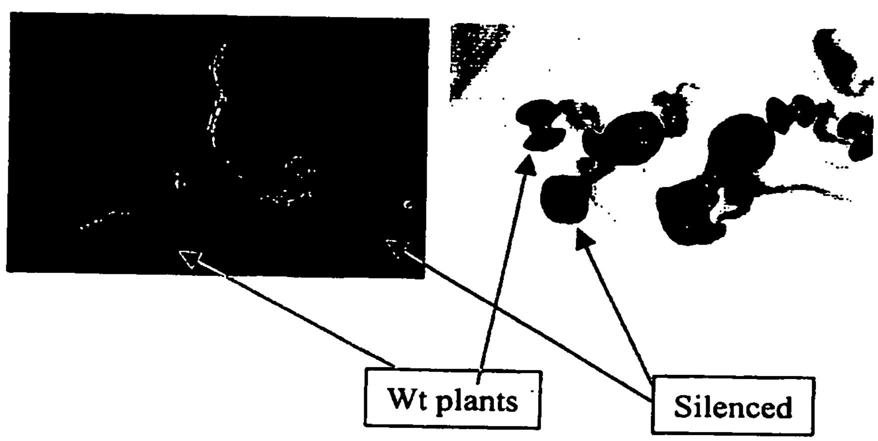


Figure 3

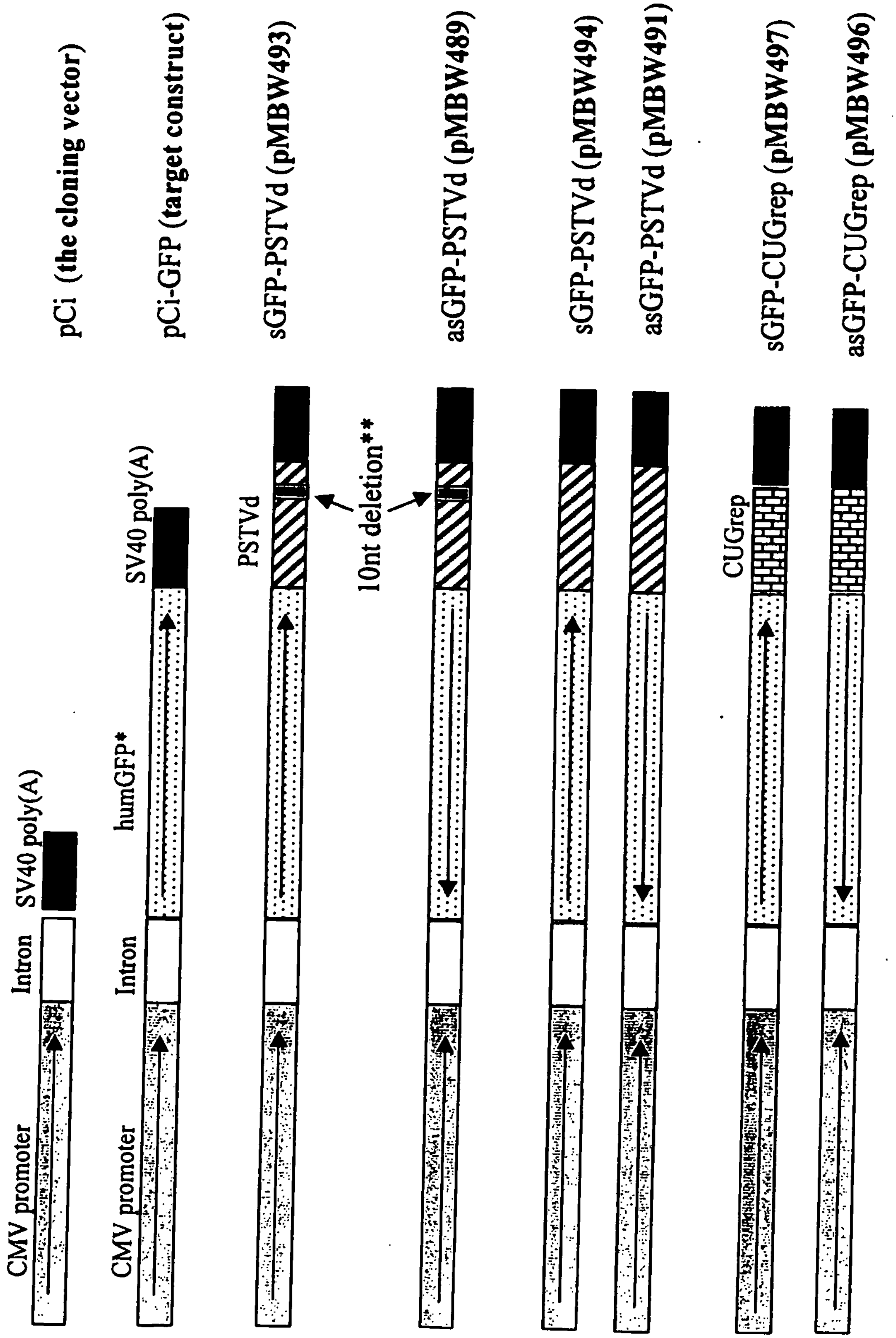
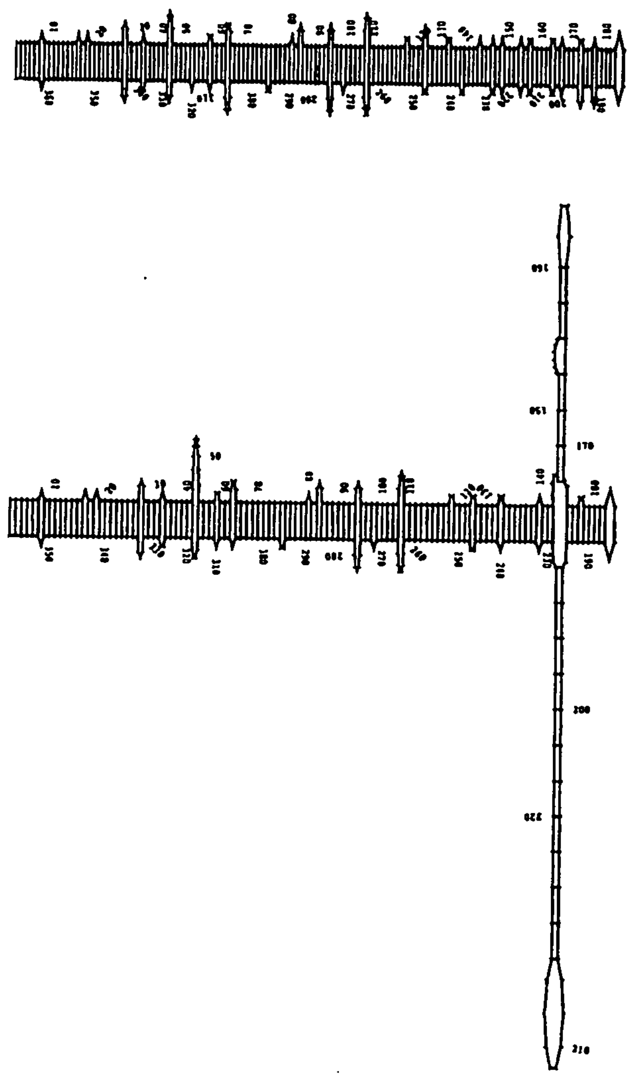

pCi (the cloning vector)
pCi-GFP (target construct)
sGFP-PSTVd (pMBW493)
asGFP-PSTVd (pMBW489)
sGFP-PSTVd (pMBW494)
asGFP-PSTVd (pMBW491)
sGFP-CUGrep (pMBW497)
asGFP-CUGrep (pMBW496)
CMV promoter
Intron
SV40 poly(A)
CMV promoter
Intron
humGFP*
SV40 poly(A)
PSTVd
10nt deletion**
CUGrep

Figure 4



A.

B.

Figure 5

INTERNATIONAL SEARCH REPORT

International application No. PCT/AU03/00292

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. 7: C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (WPIDS) AND CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, CA, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Plant Journal (2001) 27(6), Wesley et al., "Construct design for efficient, effective and high-throughput gene silencing in plants", pages 581-90	2-14, 25-37, 42-54, 83-95, 103-115, 122-134
Y	Journal of General Virology (2001) 82, Zhao et al., "Use of a vector based on *Potato virus X* in a whole plant assay to demonstrate nuclear targeting of *Potato spindle tuber viroid*", pages 1491-7	2-12, 25-35, 42-52, 83-93, 103-113, 122-132
Y	Proc. Natl. Acad. Sci. USA (1997) 94, Davis et al., "Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts", pages 7388-93	13, 14, 36, 37, 53, 54, 94, 95, 114, 115, 133, 134

[X] Further documents are listed in the continuation of Box C   [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search: 23 April 2003

Date of mailing of the international search report: 5 - MAY 2003

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C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nucleic Acids Research (2001) 29(11), Papaefthimiou et al., "Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing", pages 2395-2400	2-14, 25-37, 42-54, 83-95, 103-115, 122-134
A	Molecular Plant-Microbe Interactions (2001) 14(11), Itaya et al., "*Potato spindle tuber viroid* as Inducer of RNA Silencing in Infected Tomato", pages 1332-4	2-14, 25-37, 42-54, 83-95, 103-115, 122-134

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU03/00292

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : **1, 15-24, 38-41, 55-82, 96-102, 116-121, 135-138**

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   See supplemental box

3. ☐ Claims Nos :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: I

Claims 1, 15-24, 38-41, 55-82, 96-102, 116-121, 135-138 do not define the matter for which protection is sought in terms of the technical features of the invention (see Rule 6.3(a), Part B: Rules Concerning Chapter I of the Treaty). The specification, when read as a whole, indicates that the invention relates to the use of either viroid sequences or trinucleotide repeat sequences to target the antisense RNA to the nucleus. Claims 1, 15-24, 38-41, 55-82, 96-102, 116-121 and 135-138 are not limited to the use of such sequences and therefore do not define the subject matter for which protection is sought in terms of the technical features of the invention.